Diagnostics for Rift Valley fever virus

by

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Abstract

Rift Valley fever virus (RVFV) is a mosquito-borne, zoonotic *Phlebovirus* that is a significant threat to ruminants and humans. RVFV is categorized as an overlap Select Agent by the Department of Health and Human Services and US Department of Agriculture. Therefore, the study of RVFV's pathogenesis and the development of novel diagnostic tools for the prevention and control of outbreaks and virus spread is crucial. RVF is endemic to sub-Saharan Africa but has spread beyond the continent to the Arabian Peninsula indicating the competence of the virus to emerge in new areas. Thus, the high likelihood of RVF's spread to other non-endemic countries also spurs the need for development and implementation of rapid diagnostic tests and surveillance programs. In the US, RVFV is a Select Agent, requiring BSL-3 enhanced containment practices for research work. First, we developed a method for the detection of RVFV RNA by reverse transcriptase real-time PCR (RT-qPCR) using non-infectious, formalin-fixed, paraffin-embedded tissues (FFPET). The results from FFPET RT-qPCR were compared to prior results for fresh-frozen tissues (FFT) RT-qPCR, as well as immunohistochemistry and histopathology completed on the same FFPET blocks. We developed a novel technique using a rapid and low cost magnetic bead extraction method for recovery of amplifiable RVFV RNA from FFPET. FFPET RT-qPCR can serve as an alternative tissue-based diagnostic test, which does not require a BSL-3 research facility. Second, we assessed the diagnostic accuracy and precision of a recombinant RVFV nucleoprotein based competitive ELISA (cELISA) assay to detect RVFV antibodies. The cELISA results were compared to the virus neutralization test, the gold standard serological assay for RVFV. This prototype cELISA is an easy to implement, sensitive, specific, and safe test for the detection of antibodies to RVFV in diagnostic and surveillance applications. RVF is an important transboundary disease that should be monitored

on a regular basis. The diagnostic tests developed and validated in this thesis could be used in endemic or non-endemic countries for the early detection of RVF and assist with the implementation of countermeasures against RVFV.

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Dedication

I would like to dedicate this thesis to my mother who is always an inspiration to me.

Chapter 1 - Literature Review

Introduction

Rift Valley fever virus (RVFV) is a mosquito-borne, zoonotic pathogen of serious concern to animal and public health. RVFV is an important transboundary pathogen due to the significant potential for its international spread and use as a biological weapon[1]. It is categorized as a Select Agent by the Centers for Disease Control and Prevention (CDC) and the United States Department of Agriculture (USDA)[2]. Rift Valley Fever (RVF) is an emerging infectious disease with no therapeutic measures[1] and no fully licensed vaccine for humans. The presence of a wide range of competent mosquito vectors[3], global climate change, and international travel and trade[4] have highlighted RVF as a significant threat to both animal and public health. Therefore, study of its pathogenesis, development of effective and efficient diagnostic tools, and RVF prevention are very crucial[5]. Additionally, the increasing number of human deaths and the possibility of its transmission internationally have emphasized the need for development of therapeutics and countermeasures for the control of the disease. After the optimization and development of diagnostic tests, it important to conduct a thorough validation that provides evidence of fitness to purpose of the assay along with quality assessment[6].

RVFV virion

RVFV belongs to an order *Bunyavirales*, family *Phenuiviridae* and genus *Phlebovirus*. The RVFV virion (**Fig.1.1**) is spherical with an icosahedral symmetry and measures 90-110 nm in diameter. The virus' envelope is composed of a lipid bilayer studded with 12 nm long glycoproteins (Gn and Gc)[7]. RVFV is a tri-segmented, single-stranded, primarily negative-sense RNA virus. The three gene (tripartite) segments are designated as large (L; 6404 nucleotides),

medium (M; 3885 nucleotides), and small (S; 1690 nucleotides). The L and M segments are negative sense while the S segment is ambisense.

The L segment encodes the RNA-dependent RNA polymerase (RdRp). The RdRp (259 kDa), which is the largest protein, plays a major role in replication and mRNA synthesis[8,9]. Like influenza virus, RVFV uses a 'cap-snatching' mechanism for mRNA synthesis. For the priming of mRNA synthesis during the transcription process, the viral endonuclease activity of the RdRp cleaves the short fragments of 5'caps from host mRNA[9,10]. This process occurs in the cytoplasm[11].

The M segment encodes the major envelope glycoproteins, Gn and Gc, and two nonstructural proteins NSm (78kDa and 14kDa). A single mRNA transcribed from the M segment of RVFV translates into a polyprotein. Then the polyprotein is processed by cellular enzymes to form Gn (approximately 54 kDa) and Gc (approximately 59 kDa)[12], and at least two additional non-structural proteins, NSm (78kDa and 14kDa)[13,14]. While Gn and Gc play a crucial role in viral replication and pathogenesis, the function of NSm is not well understood[15]. Some studies have shown that NSm is not essential for either virulence[12] or viral replication in cell cultures[15]. In contrast, another study revealed that the NSm suppresses apoptosis thus, has a role in viral pathogenicity[16]. During infection, Gn and Gc play a role in viral binding and entry[17,18]. Also, they induce the production of neutralizing antibodies and elicit the protective humoral immune response[19]. Thus, these glycoproteins can be valuable targets for the development of serological tests and vaccines for RVFV.

The S segment encodes the nucleocapsid protein, Np, and a non-structural protein, NSs[20]. The Np is highly immunogenic and abundantly present in the virus. However, it only induces partially protective immunity because it cannot elicit neutralizing antibodies[5]. While Np is less

likely directly involved in pathogenesis, the NSs protein plays a critical role in RVFV pathogenesis and replication. The NSs protein is the major virulence factor of RVFV. It has antagonistic activity against interferons[21]. Also, the NSs protein induces the degradation of dsRNA-dependent protein kinase, which suppresses viral translation[22]. Though RVFV replicates in the cytoplasm, it forms filamentous structures in the nuclei of infected cells that interact with several cellular nuclear proteins, thus obstructing the cellular antiviral response[23].

The Np along with the RNA genome and viral RdRp form the ribonucleoprotein complexes (RNP)[24]. Since RVFV does not have a matrix protein, direct interaction between the glycoproteins and the RNP takes place[25].

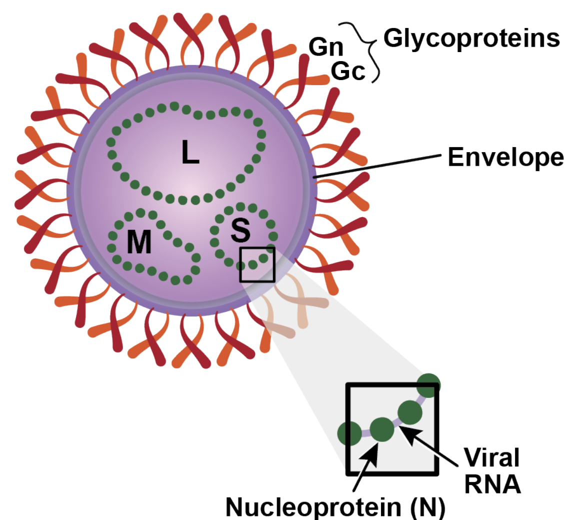

Figure 1.1 Structure of RVFV virion

Life cycle of the RVFV

The dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), which is expressed on the surface of dendritic cells has been identified as a receptor for RVFV[26]. Since RVFV has broad cell tropism, the glycosaminoglycan heparan sulfate, a polysaccharide found in animal cells may also facilitate the attachment and entry of RVFV in the

absence of DC-SIGN expression[27]. After attachment, the virus enters into the cell through caveolar-mediated endocytosis[28]. The class II fusion glycoprotein Gc, which is activated at low pH after endocytosis enables the fusion of the virion and cell membranes[29,30]. This allows the release of RNP into the cytoplasm where transcription and replication of all 3 gene segments occurs.

The RdRp plays a role in replication of each gene segment, which results in the generation of complementary RNA (cRNA). An important characteristic of the ambisense RVFV S segment RNA is that the genomic RNA also acts as cRNA. The cRNA functions as the template for the synthesis of NSs mRNA. As a result, early translation of NSs occurs and the protein plays a role in suppressing the host's innate immunity[31].

Assembly and maturation begins with the localization of Gn and Gc in the Golgi apparatus through the translocation signal peptide present on Gn[32–34]. After the recruitment of other structural proteins and the gene segments in the Golgi, mature virions form by viral budding. Then the Golgi complex undergoes morphological changes that result in many small and large vacuoles. These vacuoles help in the transport of mature virions to the cell's surface where they fuse to the plasma membrane and are released from the cell[20].

Rift Valley fever disease

In 2015, the WHO listed Rift Valley fever (RVF) among the top emerging diseases likely to cause major epidemics and in need of critical attention[35]. RVFV is a mosquito-borne *Phlebovirus* that infects a broad range of hosts including sheep, cattle, goats, buffalo, camels, and humans. The susceptibility of RVFV infection depends on livestock species, age, and viral strain[36]. The major target organs include liver, spleen and brain[37]. The clinical manifestation of RVF disease in ruminants ranges from mild, asymptomatic, self-limited illness to severe disease

including hemorrhagic fever, retinal vasculitis, hepatitis, and encephalitis[38–41]. Milder symptoms include injected conjunctiva, nasal discharge, weakness, and decreased milk production[42]. While most of the RVF cases in humans are asymptomatic and mild, a small proportion of patients have severe complications[43].

Transmission

RVFV is transmitted to livestock by mosquitoes and transmission between animals also occurs[44]. Humans acquire RVFV through numerous routes: direct contact with infected animal tissues, blood, or other body fluids; inhalation of aerosolized infected fluids; and transmission through bites of infected mosquito vectors[45,46]. More than 30 species of mosquitoes are responsible for the transmission of RVFV, however, *Aedes* spp. and *Culex* spp. are considered to be the main vectors[47,48]. The transmission cycle of RVFV involves an enzootic and an epizootic/epidemic cycle[49]. During non-excessive rainfall, the transovarian transmission of the virus to the next generation of mosquitoes plays a crucial role in order to maintain the enzootic cycle. This enzootic cycle is primarily maintained by *Aedes* spp.[50,51]. The conversion of the enzootic cycle into an epizootic cycle occurs with heavy and prolonged rainfall after which the eggs started to hatch[48,50]. *Culex* spp. are the amplifying vectors that play role in the epizootic cycle[50,52].

The risk of RVFV introduction into non-endemic regions is of great concern. The potential routes of transmission to non-endemic countries are through the movement of infected travelers and mosquitoes as well as international trade of infected animals[53]. Moreover, the presence of numerous competent vectors of RVFV in non-endemic regions increases the risk. Thus, surveillance of livestock that are imported into non-endemic regions as well as those

already present within endemic regions using validated tests is critical. Additionally, vector control programs could reduce the risk of introduction of RVFV[54].

Epidemiology

RVFV has been reported in Africa and the Arabian Peninsula. Sudden abortions in livestock following heavy rainfall are indicative of a possible RVF outbreak[46]. RVFV was first described in 1930 during an outbreak of enzootic hepatitis in a herd of ewes in the Great Rift Valley of Kenya[55]. Two large epizootics occurred during 1950-1951[56] and 1974-1976[57]. Also, there have been several sporadic outbreaks since 1950 in South Africa. A major RVFV outbreak was documented in Egypt in 1977 with 20,000–200,000 human clinical illnesses and 600 deaths[58–60] and another in Mauritania in the late 1980s[61]. The emergence of mosquito vectors due to Nile river flooding and animal trade were the suspected reasons for the outbreak in Egypt[62]. Fifteen years later in 1993, Egypt experienced a second outbreak. A key factor in this second outbreak was continuous movements of livestock[42]. Subsequently, in 2000, RVFV spread beyond Africa to Saudi Arabia and Yemen[60]. It was estimated that 40,000 animals died or were aborted and there were also 124 human deaths in Saudi Arabia[54]. RVFV epidemics were documented in several countries during 2006-2008: Somalia, Kenya, Tanzania, Sudan, Mayotte, and Madagascar[63]. In particular, the 2006 and 2007 epidemics in eastern Africa had a devastating effect in Kenya, Tanzania, and Somalia with significant livestock losses and 698 human deaths[64,65].

Several RVFV outbreak studies have been conducted. They have focused on the pattern of the outbreaks, including their association with environmental, geographical and geological factors as well as the risk factors associated with infection[66–68]. Hightower et al., 2012 concluded that the areas with heavy rainfall and flooding, poor drainage, flat landforms, and presence of

competent vectors have a high risk of occurrence of RVFV[69]. Humans who are in direct contact with infected animals are also at a high risk[45]. Therefore, continuous monitoring of competent vectors, surveillance for virus in animals, and risk analysis of the introduction of the virus into non-endemic regions are necessary.

Diagnosis of RVF

RVFV research is limited because it must be conducted in high containment laboratories[70]. Consequently, the development of safe, effective, and efficient diagnostic measures is both a challenge and an opportunity. RVFV is a hemorrhagic fever virus and listed as a category 'A' bioterrorism agent by the Centers for Disease Control and Prevention (CDC)[71]. Thus, RVFV poses a significant threat as a biological weapon, further emphasizing the need for rapid diagnosis. Several assays including the detection of the whole virus, antibodies, antigens, and nucleic acids have been developed for RVFV. Virus isolation, polymerase chain reaction (PCR), immunohistochemistry, and serological tests are the diagnostic techniques available for RVFV. According to the World Organization for Animal Health (OIE), a combination of at least two positive results from two different diagnostic tests is required for the confirmation of RVFV in animals[72]. Also, in endemic areas, a combination of clinical signs and symptoms with diagnostic tests are used for the confirmation of RVFV[63].

Virus isolation

Virus isolation can be performed using serum, plasma or whole blood collected during the febrile period (3-4 days)[73] of the disease in animals. Also, the virus can be isolated from different animal tissues (liver, spleen, and brain) at necropsy as well as aborted fetuses. Both *in vitro* and *in vivo* virus isolation can be performed. Since *in vivo* virus isolation requires intracerebral inoculation of suckling mice/hamsters, the OIE recommends *in vitro* methods using

cell lines due to animal welfare and biosafety concerns[72]. Various cell lines including African green monkey kidney cells, baby hamster kidney cells, and AP61 mosquito cells are used for *in vitro* isolation of RVFV. Consistent cytopathic effect of RVFV can be observed within 12–24 h post-infection in mammalian cell lines[74]. The combination of virus isolation with immunohistochemistry or reverse-transcription polymerase chain reaction (RT-PCR) is necessary for confirmation[72]. Virus isolation is costly, laborious, and requires biocontainment facilities for the propagation of live virus[75,76]. Thus, there is need for the development of diagnostic tools that are rapid and minimize the handling of live virus.

Viral antigen detection by immunohistochemistry

Immunohistochemistry (IHC), performed on formalin-fixed, paraffin-embedded tissues (FFPET), is a sensitive and specific technique for the detection of viral antigens in tissues, enabling visualization of RVFV antigen with histopathological context. Since 10 percent neutral buffered formalin, the standard tissue preservative, inactivates the virus, IHC can be conducted in a BSL-2 laboratory[77]. Currently, IHC in combination with histopathology is the confirmatory diagnostic tool of choice for the detection of RVFV in tissues[77,78]. In addition to its diagnostic use, IHC is used for retrospective studies.

The selection of tissue/organ sample is crucial for successful IHC[79]. Although liver and spleen are the preferred organs[78,80,81] for RVFV diagnosis, kidney, lymph node and lungs are also valuable samples for the detection of RVFV antigen[36,82–84]. Validated antibodies must be used to ensure the sensitivity and specificity of IHC. Additionally, IHC results are influenced by the quality of tissue samples[79]. Factors such as tissue collection, handling, preservation, and transportation to the laboratory need to be considered for successful IHC. Since, standardization

of sample collection, transport and IHC protocol are all needed for reliable IHC results, RVFV IHC should only be conducted in established laboratories.

Viral antigen/antibody detection by serological methods

Serological methods, either detection of antigen or antibody, are used both for surveillance of susceptible populations and outbreak management. Classical serological tests for RVFV are agar gel immunodiffusion, radioimmunoassay, hemagglutinin inhibition, and complement fixation[72]. Currently, the virus neutralization test (VNT) and enzyme-linked immunosorbent assay (ELISA) are more commonly used[85].

The VNT is considered the gold standard serological test[86]. When a comparison was made between different serological diagnostic tests for the detection of antibodies against RVFV, the VNT was discovered to have the highest sensitivity and specificity[87]. But the VNT is a costly, laborious, and time-consuming procedure with potential health risks for laboratory personnel performing the assay in biocontainment[88,89]. Therefore, ELISA offers an alternative for the detection of antibodies or antigens against RVFV.

The ELISA is a safe, rapid, sensitive, and specific test, which is used to test animals during import and export, surveillance and control programs. Also, monitoring of the immune system of animals during vaccination can be conducted using ELISA tests[90,91]. The ELISA is widely used for the detection of RVFV-specific IgM or IgG in animal or human sera. Anti-RVFV IgM antibodies can be detected from 4 days after infection and are transient. For example, Paweska et al., 2005 showed that an IgM-capture ELISA can detect RVFV antibodies within 6-22 days after onset of symptoms in naturally infected humans[89]. The detection of anti-RVFV IgM antibodies implies a recent infection. Anti-RVFV IgG antibodies can be detected from 8 days after infection and may persist for several years. Consequently, paired serum samples are

needed in order to differentiate between past and current infection[74,91–93]. There are several ELISA formats that use cell lysate or purified protein as the target antigen for the detection of antibodies against RVFV[89,91,94,95]. Also, ELISAs based on recombinant nucleoprotein (recNp), which does not require a biocontainment facility for their production or use, have been reported[75,88].

Detection of RVFV antigen using ELISA could be an alternative for detection of antibodies against RVFV because the antibodies against the virus may not be detectable during the first few days of infection[96,97]. However, the assays for detection of viral antigen lack sensitivity[92] and require reagents that are costly and challenging to produce[75]. Due to these limitations of antigen detection ELISAs, IgM or IgG ELISAs for the detection of antibodies against RVFV are widely used[92].

With the limitations of classical serological methods, the ELISA technique is in wide use for the detection of antibodies. However, lack of international standardization, lack of inter-assay consistency, and full validation of ELISA techniques have been challenges to the use of ELISA[98]. Although ELISA is more sensitive than classical methods for the diagnosis of RVFV, non-specific binding and cross-reactivity with other *Phleboviruses* is a major concern[91]. Thus, complete validation of each ELISA test is crucial before using it as a diagnostic or surveillance test.

Nucleic acid detection by PCR

Some molecular methods, specifically the detection of viral RNA, are rapid and economic techniques for the detection of RVFV genome, and therefore are an excellent alternative to virus isolation and serological methods. They are valuable for effective management of outbreaks because they can detect the presence of viral RNA rapidly enabling

swift application of appropriate control measures[99]. Various rapid and accurate molecular methods have been developed and additional studies are underway for the development and evaluation of molecular diagnostic tests.

Several reverse transcriptase polymerase chain reaction (RT-PCR) and real time RT-PCR (RT-qPCR) methods have been developed[100–104]. These methods have a limit of detection between 10 and 100 genome copies[105] and are recommended by OIE[72]. While expensive equipment is required to conduct RT-qPCR, it is the most commonly used technique for the detection and quantification of RVFV RNA due to its high analytical sensitivity and specificity[106]. Also, when compared with conventional RT-PCR, RT-qPCR is a rapid, standardized, quantitative method with less chance of contamination.

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) and recombinase polymerase reaction (RPA) are simple and sensitive techniques. These techniques use an isothermal reaction, which is feasible for rapid detection in the field. Since an outbreak of RVFV can occur in remote areas and can become catastrophic within a short time period, the development and evaluation of field deployable molecular techniques would be useful to diagnose and employ control measures effectively[63]. Although six sets of primers are needed for RT-LAMP, it is a single step, rapid, simple, and cost-effective assay for the amplification of L segment of RVFV[107,108]. Moreover, RT-LAMP is a sensitive and specific assay comparable to RT-qPCR[105]. Similarly, RPA is also a rapid and sensitive assay that uses primers and probes similar to those used for RT-qPCR in contrast to complex primers used for RT-LAMP. However, RPA requires a portable fluorescence reader for field use[109].

Ebola, Marburg, Lassa, Dengue, and Yellow fever viruses like RVFV are the important causative agents of viral hemorrhagic fevers (VHFs). Considering that VHFs have common signs

and symptoms and are clinically challenging to differentiate, multiplex RT-PCR assays have been developed for simultaneous detection of multiple pathogens[110,71]. These assays are important for accurate and rapid diagnosis needed to control spread and apply appropriate treatment, particularly in areas where multiple VHF pathogens are endemic[111].

Molecular methods are sensitive but variation in results can occur in different laboratory settings. Thus, a standardized protocol and controls are required when performing the test to reduce this variation as well as detect contamination issues[99]. During RVF outbreaks, molecular methods are particularly beneficial for the detection of RVFV RNA at early infection time-points when antibodies cannot be detected[101]. Therefore, molecular methods are useful for early diagnosis in order to control and prevent the spread of RVF disease. However, the need for specialized instrumentation and skilled laboratory personnel as well as the short time period of viremia pose challenges for the use of molecular methods for the detection of RVFV RNA, especially for outbreaks in remote regions[75].

Diagnostic Assay Validation

Validation of an assay is a series of inter-related processes that are conducted to determine the competence of well-developed and optimized assay for an intended purpose[112]. It is critical for any developed assay to have a complete validation before its commercial use. A fully validated assay has the capability of identifying an analyte present in the given sample and classifying the animal as positive or negative[113]. Several factors need to be optimized and tested during the assay development and validation pathways. According to OIE[6], after the development and optimization of an assay, the validation pathways comprise of four stages; Stage 1: analytical characteristics; Stage 2: diagnostic characteristics; Stage 3: reproducibility; Stage 4: implementation. Along with the development and validation pathways, monitoring of

the assay performance after initial validation is critical, requiring continuous monitoring, evaluation, and maintenance of the assay.

RVFV vaccines

It has been more than eight decades since the discovery of RVFV[82], but the development of a safe and efficient vaccine for human and animals is still a challenge[114]. Moreover, there are no specific therapeutic measures available for RVF[74]. A vaccine is a modified form of a natural immunogen. It may be either the whole pathogen, one of its components, or a toxin. Ideally, it should be easy to administer, single dose, provide long-term immunity, unable to revert to virulent virus, stable at room temperature, and cost-effective. Additionally, vaccines that allow the differentiating of infected animals from vaccinated (DIVA) are important for international trade and surveillance[115]. Strategies tried for RVFV vaccines include live virus attenuation, virus inactivation, virus-like particles, recombinant viral vectors and DNA vaccines[5]. Three licensed veterinary RVF vaccines (Smithburn vaccine, a formalin-inactivated vaccine derived from Entebbe strain, and Clone 13) are available for use in endemic regions. In the US, live-attenuated mutagenized passage-12 (MP-12) vaccine strain is conditionally licensed for animal vaccination[116].

The Smithburn strain, a live attenuated vaccine developed in 1940, was the first RVF vaccine[117]. Although Smithburn strain is still being used, it is not safe for young animals or gestating adults. Moreover, the risk of reversion of this vaccine strain to full virulence drove the need for alternative vaccines[118,119]. Consequently, two other live attenuated vaccines, MP-12 and Clone 13 were developed.

Clone 13 is a naturally attenuated RVFV isolate with a large deletion in the NSs gene[120]. When this vaccine was first evaluated in sheep, effective protection with no pathogenic side

effects and no abortions in pregnant ewes was observed[121]. In contrast, a recent study concluded that Clone 13 can cross the sheep placental barrier causing fetal infections, malformations, and stillbirths[122].

MP-12 was produced by serial passaging of the Zagazig Hospital 548 (ZH548) RVFV strain 12 times in the presence of mutagen 5-fluorouracil. The vaccine is considered to be effective in sheep and cattle[5]. A human phase II clinical trial was conducted for MP-12 vaccine in which no reversions of vaccine virus (in attenuated regions) to wild-type RVFV occurred as confirmed by genetic analysis[123]. However, the mechanisms of MP-12 attenuation are still not fully understood[114,124]. Also, in a study in pregnant ewes, MP-12 caused teratogenic effect, malformations, and abortion in sheep[125] and in another experimental study, 6 out of 10 four-month old calves inoculated with MP-12 had multifocal, hepatic necrosis and one calf's liver was positive for RVFV antigen by IHC[126].

Finally, no licensed vaccine for humans is available, in the US, RVFV TSI-GSD-200 is used for military and at-risk laboratory personnel and is provided by the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID). Unfortunately, it is an inactivated vaccine that requires multiple inoculations and enrollment in a costly clinical trial program[127]. Since humans typically get infected after contact with infected animals, effective vaccination of ruminants can also help prevent RVF in humans. Overall, there is significant room for improvement in licensed vaccine options and therapeutics for RVF.

FFPET as a diagnostic specimen

FFPET has been extensively used for histopathology and IHC for the diagnosis of many infectious diseases. FFPET archives serve as an invaluable source of information for molecular genetics research[128,129]. Recent studies have also revealed that FFPET could be a potential source

of material for retrospective analysis using RT-qPCR and microarrays techniques[130,131]. When compared with fresh frozen tissue as a specimen for analysis, FFPET is cheaper to store for a long period of time, stable at room temperature, and easier to process. Moreover, the retrospective investigation of etiology and epidemiology of disease can be conducted using FFPET[131,132].

Unfortunately, nucleic acid obtained from FFPET is of low quality and it is harder to extract amplifiable RNA/DNA from FFPET in comparison to fresh-frozen tissues[132–135]. The preservation process for FFPET specimens involves 10% neutral buffered formalin fixation, for at least 24 h, typically followed by a 12 h long procedure in an automatic processor using formalin, various graded alcohols, xylenes, and paraffin infiltration then finally, embedding in paraffin. These aforementioned processes induce fragmentation of the nucleic acids and formalin specifically induces chemical modification of the RNA/DNA by addition of monomethylol (-CH2OH) group to the nucleotides of RNA as well as cross-linking of nucleic acids (NA) with proteins[136–140]. The methylol addition inhibits the reverse transcription process decreasing the sensitivity of RT-PCR[136]. The crosslinking between NA and proteins causes restrictions during the extension of the primer[137]. Thus, the attainment of sufficient and stable RNA from FFPET is challenging. In spite of the technical challenges, FFPET is used as a specimen to study tumor gene expression[128,129,141] as well as for the detection of viral RNA[142–144].

Although several studies have been conducted using FFPET, there is no standard protocol for the deparaffinization and extraction of nucleic acids[145,146]. There are many factors that affect FFPET PCR results including the age of FFPET blocks, degree of tissue autolysis prior to preservation, fixative quality, fixation time, tissue type, and nucleic acid extraction procedure[147–149]. Thus, standardization and optimization of protocols are necessary before it will be possible to

obtain the same quality and quantity of nucleic acids from FFPET in different laboratory settings.

Gaps in Knowledge

Definitive diagnosis is critical for RVF and there are only a few validated assays available. FFPET has been used for detection of RVFV using histopathology in combination with IHC technique, however, to our knowledge it has not been successfully used for further molecular analysis. A study was conducted to purify suitable RVFV RNA from archived FFPET for gel-based reverse transcriptase PCR (RT-PCR) and next generation sequencing but no RT-PCR amplicons were achieved[150]. A reliable method for the quantitative detection of viral RNA from RVFV infected FFPET does not exist.

Serological tests have always been one of the easiest methods for the diagnosis of infectious disease. ELISA tests using recNp offer a safe, reliable, effective, and efficient alternative technique. They do not require handling of live virus and their production and use can be conducted outside of biocontainment facilities[151]. While a prototype recNp cELISA had been developed for the USDA Animal and Plant Health Inspection Service (APHIS) National Veterinary Stockpile, it was not yet validated.

Purpose of research

We addressed the aforementioned gaps in RVFV diagnostics. First, we developed and validated a protocol for sensitive and specific extraction of RVFV RNA from FFPET. The protocol uses a rapid, low cost magnetic bead nucleic acid extraction enables sensitive detection of RNA specific for RVFV and for PCR we used the Wilson et al., 2013 RVFV multiplex PCR[152]. Next, we determined the diagnostic accuracy and precision of the aforementioned RVFV recNp cELISA. Both of these techniques are at OIE stage 2 of validation and required the

inclusion of field samples from endemic countries to move to stage 3. With further validation, these tests should contribute to the detection of RVFV and help in prevention and control of RVFV during outbreaks. Additionally, the RT-qPCR technique using FFPET should be suitable for retrospective molecular analysis studies of RVFV.

Chapter 2 - Detection of Rift Valley Fever Virus RNA in Formalin-Fixed, Paraffin-Embedded Tissues using Reverse Transcriptase Real Time PCR

Preface

This is a draft manuscript that will be further refined and submitted for peer review under the title:

Detection of Rift Valley Fever Virus RNA in Formalin-Fixed, Paraffin-Embedded Tissues using Reverse Transcriptase Real Time PCR

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Introduction

Rift Valley fever virus (RVFV), a mosquito-borne zoonotic pathogen (genus *Phlebovirus*; family *Phenuiviridae;* order: *Bunyavirales*)[153], is endemic to sub-Saharan African countries[73]. It is an enveloped, single-stranded, ambisense polarity RNA virus with three gene segments designated as L (large; 6404 nucleotides), M (medium; 3885 nucleotides), and S (small; 1690 nucleotides)[154]. RVFV is the causative agent of Rift Valley fever (RVF), a disease, which has devastating effects on both livestock and human health. It causes abortions and death in ruminants and illnesses ranging from fever, encephalitis to death in humans[155].

Since RVFV's first isolation in Kenya in 1930[55], there have been many outbreaks reported in several countries including Kenya, Tanzania, Somalia, South Africa, Madagascar, Egypt, Sudan, Mauritania, Senegal, Saudi Arabia, and Yemen[59]. The increasing number of human deaths in more recent outbreaks, lack of licensed vaccines, the possibility of its spread to non-endemic countries, and the classification of RVFV as a Select Agent in the United States all emphasize the need for further study of RVFV's pathogenesis and the development of diagnostic tools and medical countermeasures for RVF[5,156]. However, research on RVFV is limited because it requires BSL-3 research facilities, in particular, BSL-3Ag for research with ruminants[70]. To overcome this issue, the potential use of formalin-fixed, paraffin-embedded tissues (FFPET) for the detection of RVFV RNA using reverse transcription polymerase chain reaction (RT-PCR) for diagnostics and for conducting retrospective research is promising[77]. The use of FFPET for immunohistochemistry (IHC) in combination with histopathology is the accepted tissue-based confirmatory test of choice for RVFV during epidemics[77]. While fresh and frozen tissues (FFT) remain the ideal source for molecular diagnosis, FFPET is an attractive alternative research

material because it is safer to process and cheaper to store for a long period of time[131]. Moreover, FFPET is a better choice in the absence of a cold chain.

Attaining sufficient and stable RNA from formalin-fixed tissues is challenging because formalin degrades and chemically modifies the RNA by addition of monomethylol (-CH2OH) group to RNA followed by cross-linking of nucleic acids (NA) with proteins[136,145,157]. Consequently, quality and quantity of nucleic acid (NA) obtained from FFPET will be low in comparison to FFT. Despite these limitations, FFPET is used for gene expression analysis to study changes during tumor progression[128,129,141]. FFPET has been used for the detection of infectious diseases including West Nile virus, enterovirus, and hepatitis C virus by RT-PCR[142–144] and high-throughput RNA sequencing of archival autopsy lung samples from 1918 pandemic influenza victims[158]. Several studies have shown that detectable RNA can be extracted successfully by introducing various modifications to the extraction process. By increasing the deparaffinization temperature, proteinase K concentration and incubation time, RNA was recovered from FFPET infected with BSL-3 and -4 pathogens using a Paraffin Block RNA isolation kit (Ambion, Texas, USA)[145]. Another successful study used the Optimum RNA isolation kit (Ambion) with modifications for the extraction of RNA from formalin-fixed paraffin-embedded cell pellets[159]. Unsurprisingly, since no standardized protocol for the extraction of RNA from FFPET exists, there is wide variability in the quality and quantity of RNA extracted[149]. In short, given a successful RNA extraction method, further downstream molecular analysis can be conducted using FFPET[131].

While the literature reports studies that use the extraction of RNA from FFPET followed by quantitative PCR analysis, to our knowledge a reliable method for the quantitative detection of viral RNA in RVFV infected animal tissues does not exist. A recent study attempted to purify

usable RVFV RNA from archived FFPET for gel-based RT-PCR and next-generation sequencing[150]. However, success was limited, and no RT-PCR amplicons were recovered for the target, a 490-nucleotide portion within Gn's encoding gene. Thus, our objective was to develop and evaluate a protocol using FFPET for sensitive and specific detection of all three RVFV RNA segments. We compared automated magnetic bead extraction, an easy, rapid, and economic[160] method, to spin column extraction, to obtain the RNA from FFPET.

Materials and Methods

Formalin Fixed Paraffin Embedded Tissues

Table 2.1 lists all the RVFV infected archival FFPET blocks included in this study. These blocks, from prior experimental animal studies[155,161,162], were assembled to represent multiple tissues types (liver, spleen, lung, lymph node, and kidney) for which RVFV is tropic, two virulent RVFV strains (Saudi Arabia 2001-1322 (SA01)[163] and Kenya 2006-128b-15 (Ken06))[164] and two susceptible hosts (cattle and sheep). All animal studies were conducted in the Biosecurity Research Institute's biosafety level 3 agriculture (BSL-3Ag) facilities in Manhattan, Kansas. A total of 82 FFPET samples (cattle=36, sheep=34, goat=12) from animals born and raised in the United States, a RVFV free country, were included in this study as negative RVFV controls. Out of 82 samples, 48 FFPET samples were taken from a previously conducted Schmallenberg virus cattle and sheep (cattle=24, sheep=24) study. The goat tissues, provided by co-author Jessie Trujillo, were from a caprine encephalitis and arthritis study.

All tissues were fixed in 10% neutral buffered formalin (NBF) for a minimum of 7 days, trimmed, processed, and embedded in paraffin using standard techniques. Briefly, formalin-fixed tissues were trimmed into 3-4 mm thick pieces and placed into labeled cassettes. These cassettes were then placed into an automatic processor (Sakura Finetek, Alphen aan den Rijn, The

Netherlands). The 12 h processing procedure included 3 h in 10% NBF, 30 min in 70% ethanol, 30 min in 80% ethanol, 90 min in 95% ethanol, 90 min in 100% ethanol, 90 min in xylenes, and 2h of paraffin infiltration. Post-processing, samples were embedded in Parapath X-tra paraffin (Leica Biosystems Inc., IL, USA). Using a fresh microtome blade for each block, five 4-5 µm thick tissue sections were cut for each sample and stored in sterile tubes at -80° C until deparaffinization.

Deparaffinization

In a clean type II biosafety cabinet, with the airflow turned off, 4-5 µm FFPET sections were carefully transferred to 320µl pre-aliquoted deparaffinization solution (Qiagen, Hilden, Germany) using sterile Adson-Brown tissue forceps and vortexed for 10 sec. The tubes were then incubated in a pre-heated Vortemp 56 shaker/incubator (S2056A, Labnet International, New Jersey, USA) at 56° C for 5-6 min at 80 rpm. After cooling at room temperature for 2-3 min, 180 µl of alkaline tissue lysis buffer (Qiagen) was added and the samples were vortexed with centrifugation for 1 min at 10,000 rpm. For reversal of crosslinking, 40µl proteinase K (Qiagen) was added to the lower phase at the bottom of the tube and the samples were incubated at 56°C for 1 h at 10 rpm in a pre-heated Vortemp 56 shaker/incubator (Labnet). The samples were removed and incubated at 80°C for 35 min at 10 rpm in the preheated Vortemp 56 shaker/incubator (Labnet). The lower, clear phase was transferred to 320 µl pre-aliquoted RLT lysis buffer (Qiagen) filled tubes. RLT buffer contains a high concentration of chaotropic salts that support the binding of RNA to the silica membrane[160]. The deparaffinization step generates tissue lysate in RLT buffer. The lysates were immediately stored in -80°C until use in the next step, NA extraction.

Nucleic Acid Extraction

We compared both bead and column methods for NA extraction from FFPET lysates. These extractions were done side by side, comparing the GeneReach DNA/RNA extraction kit for an automated magnetic bead extraction on either the Taco Mini (tm0057, GeneReach Biotechnology Corp., Taichung City, Taiwan) or the Biosprint 96 (Qiagen) to a silica-based membrane and microspin technology (Qiagen RNeasy Mini Kit, Hilden, Germany) with modifications for column extraction. One hundred µl of lysate was used throughout with the exception of a small additional experiment in which bead extraction with 200 µl lysate was examined. All FFT RT-qPCR results were from prior studies[155,161,162]. Universally, this FFT NA was purified using the magnetic-bead capture MagMAX-96 total RNA Isolation kit (Life Technologies, NY, USA) as described previously[152].

Real time PCR (RT-qPCR)

RVFV Multiplex Reverse Transcriptase Real Time PCR (RT-qPCR) on FFT was performed as described previously (Wilson et al., 2013). Briefly, the L, M, and S segments were detected using AgPath RT-qPCR mix on the Stratagene MX300P thermocycler (Agilent Technologies, Inc., CA, USA)[155,161,162]. The FFPET RT-qPCR used qScript XLT 1-Step RT-qPCR ToughMix (Quanta BioSciences Inc., Massachusetts, USA) on the Biorad CFX thermocycler (CFX96 Optics Module, Bio-Rad Laboratories Inc., CA, USA). The thermocycler conditions were: 50°C for 20 min, 95°C for 5 min, 95°C for 10 sec, 60°C for 1 min followed by 45 cycles of 95°C for 10 sec.

Limit of Detection and RNA Copy Number Determination

Ten-fold serial dilutions of quantitated RVFV L, M, and S *in vitro* transcribed (IVT) RNA were used to generate an eight-point standard curve using three PCR well replicates per

dilution. Additionally, similar standard curve data for viral RNA was considered in determination of thresholds (Trujillo et al., unpublished). For FFPET RT-qPCR, an individual PCR replicate was considered positive if its threshold cycle (Ct) was < 38. This Ct cut-off of 38 corresponded with the desired 50% limit of detection (LOD50)[165] as determined by the aforementioned standard curve results.

For all FFPET RT-qPCR, three replicates for each sample were run in multiplex. Each gene was reported as positive, if at least 2/3 PCR replicates had a Ct <38 and suspect if 1/3 PCR replicates had a Ct < 38 or at least 2/3 PCR replicates had a Ct ≥38. The samples were categorized as positive if at least 2/3 genes were positive. The samples were categorized as suspect if 1/3 genes were positive or if any genes were suspect. We also always included an extraction negative control and 10% of each plate consisted of no template control in order to evaluate the true negative RT-qPCR results in the study.

Gene copy number (CN) was calculated using IVT RNA standard curves for each gene segment. The RVFV CN for all positive genes was mathematically determined using the formula y=mx+b where x= copy number, y= the PCR-determined mean threshold cycle (Ct) for each gene segment, m is the slope of the standard curve and b is the y axis intercept[166]. CN was expressed as log base 10 for each of the gene segments. Mean increases in CN for sheep and cattle samples were determined by taking the difference in CN for each gene segments followed by overall average estimation and then converted to log base 10.

Histopathology

In prior published studies, hematoxylin and eosin (H&E) stained liver tissues of cattle[161] and sheep[155,162] were examined microscopically and semiquantitatively scored from 0 to 4, where 0=no lesions attributable to Rift Valley fever virus; 1=multifocal, mid-zonal to central foci of

lymphohistiocytic inflammation with lesser numbers of plasma cells and occasional single hepatocyte apoptosis; 2=multifocal, 1-2 mm areas of mid-zonal to central lymphohistiocytic inflammation frequently with central necrosis shifting inflammation to predominantly neutrophils, less than 5% of examined parenchyma involved; 3=as prior but more severe necrotic lesions involving up to 15% of hepatic tissue reviewed, additionally present is scattered hepatocyte apoptosis; 4=greater than 15% of the parenchyma is necrotic and severe multifocal hemorrhage is also present. Lesions attributable to RVFV observed in other tissues were described[155,161,162]. The data from these previous studies was referenced for comparison in this study.

Immunohistochemistry

Prior IHC result were used for all liver samples, both cattle and sheep, as well as all non-liver sheep samples[155,161,162]. For all non-liver cattle samples, IHC was performed *de novo* for this research. For all IHC four-five-µm thick tissue sections placed on positively-charged slides were deparaffinized using xylenes, rehydrated in graded alcohols (100%-70%) to distilled water, antigen retrieved using sodium citrate buffer (pH 6.0) in a vegetable steamer for 20 min and cooled for 15 min at room temperature. Unless otherwise noted, tissues were washed in Tris-buffered saline with 0.01% tween-20 added between each step. Tissues were blocked with 3% hydrogen peroxide diluted in distilled water and 5% secondary antibody matched serum for 10 and 30 min respectively. In the prior studies, for the detection of RVFV nucleoprotein in cattle liver[161] and all sheep[155,162] tissues, rabbit polyclonal anti-RVFV nucleoprotein antibody[78] was diluted 1:500-1:1000 in Dako antibody diluent (S3022, Dako, Agilent, Santa Clara, US) and incubated with the tissues at 4°C overnight. In contrast, for the detection of RVFV nucleoprotein antigen in all non-liver cattle tissues, a mouse monoclonal anti-RVFV nucleoprotein antibody

(MAB240P, Maine Biotechnology Services, Portland, USA) diluted 1:500 in Dako antibody diluent (S3022, Dako, Agilent) was used and incubated overnight at 4°C. After primary antibody incubations, a matched secondary and avidin-biotin complex detection reagents were applied per manufacturer's instructions, Vectastain Elite ABC kits (PK6101 and PK6102, VL), 3,3' Diaminobenzidine (DAB) (VL) chromogen was applied, slides were washed with distilled water and Mayer's hematoxylin (Electron Microscopy Sciences (EMS), PA, USA) counterstain was applied. The slides were dehydrated and mounted with Permount (EMS). All IHC, both prior studies and this one, was conducted in the same laboratory and historically, IHC with the anti-RVFV nucleoprotein rabbit polyclonal has yielded equivalent positive/negative results to IHC with the monoclonal.

RVFV viral antigen IHC was semi-quantitatively scored similarly in both sheep and cattle liver from 0 to 3, where 0=no positive labeling; 1=single cell to scattered multifocal cytoplasmic hepatocyte signal; 2=up to 10% of hepatic parenchyma is positive for RVFV antigen; 3=as prior but more extensive, greater than 10% of liver tissue reviewed. While non-liver tissues were called positive/negative for viral antigen. Prior study data[155,161,162] were referenced here and the same scoring system was applied to additional IHC conducted specifically for this study. In this way, new IHC results were normalized to referenced results. The same veterinary pathologist read the histopathology and IHC for all studies. Images were captured with a DP27 camera (Olympus, Tokyo, Japan) on a BX46 light microscope (Olympus) using CellSens Standard Version 1.16 (Olympus). All microscopic images were further color calibrated using ChromaCal software ver 2.5 (Datacolor Inc., Lawrenceville, NJ, USA) as per the manufacturer's instructions and the figure panels were composed in Adobe Photoshop and InDesign CC 2018 (Adobe, CA, USA).

Analysis

Mean Ct values and standard deviations for FFPET RT-qPCR results were calculated. Inter-assay variability was determined by calculating the percentage of coefficient of variation (CV=standard deviation/mean) using mean Ct value from RT-qPCR. Two samples (4 aliquots of sample #17 and 9 aliquots of sample #19) with different Ct values were used to test the inter-assay variability during extraction of RNA. The testing of each individual aliquot was done on a separate day by the same operator. The sensitivity of the test was calculated using the formula; TP/(TP+FN) and specificity as TN/(TN+FP), where TP is the number of true positives; FN is the number of false negatives, TN is the number of true negatives, and FP is the number of false positives. All the descriptive statistics were generated using Microsoft Excel 2007 unless stated otherwise.

Cohen's Kappa statistic and McNemar's test for paired data were used to determine the agreement beyond due to chance and test the proportion of positive results obtained between the IHC, FFT RT-qPCR and FFPET RT-qPCR tests respectively. The scale of Landis and Koch[167] was used to interpret the agreement between tests, as follows: <0 poor agreement, 0.01 to 0.2 slight, 0.21 to 0.40 fair, 0.41 to 0.6 moderate, 0.61 to 0.80 substantial and 0.81 to 1.0 almost perfect agreement. A non-significant McNemar's test ($P < 0.05$) indicates there is little evidence that the proportion of positives differs between tests[168]. We analyzed these data using STATA 12 software (StataCorp LP, Texas, USA).

Results

Assay specificity

The specificity of the FFPET RT-qPCR assay for both the spin column extraction and magnetic bead extraction were determined to be 100% (95% CI = 95.6 -100%) using known RVFV negative FFPET cattle, sheep, and goat samples.

Sensitivity of FFPET RT-qPCR compared to FFT RT-qPCR

We used archival results for 29 FFT RT-qPCR positive samples (sheep=13 and cattle=16)[155,161,162] for the qualitative comparison of RVFV RNA detection by FFPET RT-qPCR to FFT RT-qPCR. As seen prior with FFT RT-qPCR, FFPET RT-qPCR readily detected viral RNA in day 3 post-inoculation infected (earliest time-point available) sheep and cattle tissues. Both techniques performed similarly well on late post-inoculation time-points. The sensitivity of detection of RVFV RNA by FFPET RT-qPCR in comparison to FFT RT-qPCR for the sheep samples was 100% (95% CI, 75.3-100%) (Table 2.2). However, for cattle samples the sensitivity was only 75% (95% CI, 47.6-92.7%) (Table 2.3). Out of the 16 cattle samples that were positive by FFT, 3 samples #25, #28, #41 were suspect, and a sample #30 was negative by FFPET RT-qPCR.

Effect of increasing the lysate volume

In order to examine if increasing the lysate volume used in the FFPET bead extraction step would increase assay sensitivity, the aforementioned negative and suspect samples (Table 2.3) were run with 7 other diverse result (positive, suspect, and negative) cattle and sheep samples. Overall, increasing the volume from 100 to 200 µl of FFPET lysate increased the sensitivity of detection of RVFV RNA from 36.4% to 54.5%. Two originally suspect samples

#25, #38 were detected as positive and two originally negative samples #30, #39 changed to suspect (Table 2.4).

FFPET RT-qPCR detection of RVFV RNA in comparison to IHC and histopathology

FFPET RT-qPCR results were compared to IHC results for 20 liver and 23 non-liver FFPET samples. The overall sensitivity of detection of RVFV RNA by FFPET RT-qPCR in comparison to IHC was 89.5% (95% CI, 75.2-97.1%). For all the liver tissues (sheep and cattle), the sensitivity of detection of RVFV RNA by FFPET RT-qPCR in comparison to IHC was 100% (95% CI, 80.5-100%) (Table 2.5). For an IHC score of 2, on average FFPET RT-qPCR Ct values were 26.7, 27.3, 28.6 with standard deviations of 3.7, 3.8, 4.3 for the L, M and S respectively. For an IHC score of 3, average Ct values were 21.3, 21.6, 23.5 with standard deviations of 2.1, 2.2, 2.2 for L, M and S respectively. As would be predicted, the average Ct value for each gene segment decreases with an increase in IHC score. When FFPET RT-qPCR detection of RVFV RNA for all liver samples was compared to histopathology results, there were only 3 inconsistent samples #30, #39, #40 (Table 2.5). All of these were negative for viral antigen by IHC (score=0) and two, #39 and #40, scored a 1 for histopathology, while #30 scored a 2.

The sensitivity of detection of RVFV RNA by FFPET RT-qPCR in comparison to IHC for all the non-liver tissues (sheep and cattle) was 80.9% (95% CI, 58.1-94.6%). The non-liver FFPET RT-qPCR and IHC results correlated with the exception of six samples: #4, #25, #28, #29, #38, and #41 (Table 2.6). Four of these samples (#25, #28, #29, #38) were suspect by FFPET RT-qPCR but positive by IHC and samples #4 and #41 were suspect by FFPET RT-qPCR and negative by IHC. As mentioned prior, when the lysate volume was increased, 2 of these samples (#25 and #38) became FFPET RT-qPCR positive. Spleen sample #28 and kidney

sample #29, both positive by IHC, were from a single 4 days post-infection SA01 infected calf for whom the liver histopathology score was a 2 but no viral antigen was detected by IHC. Scant viral antigen was seen in the spleen and only present in focal pathology in the kidney.

Comparison of the automated magnetic bead and spin column extractions from FFPET

Positive/negative determination for the detection of RVFV RNA from FFPET with both extraction methods was similar (Table 2.7 and 2.8). Out of the 43 samples, results differed for three samples (#4, #25, and #40). Spleen sample #25, positive by FFT RT-qPCR with Ct range of 29 to 34, was detected positive by FFPET column extraction but suspect by bead extraction. Interestingly, while sample #4 was negative by column extraction it was suspect by bead extraction. Sample #40 was suspect by column extraction but negative by bead extraction. An increase in CN for L, M and S segments for both sheep and cattle samples was observed for column compared to bead extraction (Table 2.7 and 2.8). On average, for sheep samples, there were 5.9, 5.7, 6.9 log increases in CN for the L, M and S segments respectively. Similarly, for cattle samples, 5.6, 5.3, 5.9 log increases were observed for the L, M and S segments respectively.

Inter-assay variability during extraction of RVFV RNA

Samples #17 and #19 (both sheep spleen), used to determine the inter-assay variability, were strongly positive by FFPET RT-qPCR (19-22 Ct) and also positive by IHC and FFT RT-qPCR. The CVs for the L, M, and S segments of sample #17 were 6%, 6%, 8% respectively (Table 2.9). Similarly, the CVs for L, M, and S segments of sample #19 were 7%, 6%, 6% respectively (Table 2.9).

Agreement between the tests

Statistical analyses using McNemar's test showed no significant difference ($P > 0.05$) between the positive proportions of IHC, FFPET RT-qPCR using bead extraction and column extraction and FFT RT-qPCR using bead extraction methods[168]. The extent of agreement between the tests beyond chance was considered moderate to almost perfect between these tests (IHC, FFPET RT-qPCR using bead and column extraction, and FFT RT-qPCR) according to the Landis and Koch scale (from 0.52 to 0.93) (Table 2.10). Substantial agreement was observed between FFPET RT-qPCR for both bead and column extraction methods when each of them were compared with FFT RT-qPCR using bead extraction method ($k > 0.6$).

Discussion

Here we report the first successful application of formalin-fixed, paraffin-embedded tissues (FFPET) RT-qPCR for the detection of RVFV. Use of FFPET as the specimen for molecular analysis is challenging and has not been extensively used in virology in comparison to fresh and frozen tissues. Due to the low quality of the nucleic acid (NA) typically extracted from the FFPET[169], its use for molecular detection of pathogen RNA is under-investigated. In a previous RVFV study, Mubemba et al. attempted to amplify a 490-nucleotide sequence within the Gn gene of RVFV from FFPET using conventional and real-time PCR methods but were unsuccessful[150]. In contrast, our study using FFPET from cattle and sheep experimentally infected with RVFV demonstrates that RVFV RNA (L, M, and S gene segments) can be detected using RT-qPCR.

The quality and quantity of RNA extracted from FFPET depends on several factors including fixation process and its duration, embedding, storage time and conditions, and optimization of methodologies[147–149]. Extraction of useable RNA from FFPET is challenging due

to formalin's chemical modification of nucleic acids, which includes both protein-NA cross-linking as well as NA fragmentation. However, several studies have shown that protocol optimization improves RNA yields from FFPET[145,149,170]. Here, we doubled the volume of FFPET lysate used for extraction to increase the sensitivity of detection of RVFV RNA. Two samples originally reported as negative on FFPET RT-qPCR became suspects and another two samples originally reported as suspects on FFPET RT-qPCR became positive with this increase in lysate volume (Table 2.4). Further protocol optimizations may yield even better results, particularly for cattle.

While our comparison of RVFV RNA detection from FFPET and FFT showed that the sensitivity of detection of FFPET RT-qPCR was 100% for sheep samples, the sensitivity decreased to 75% for the cattle samples. Out of the 16 cattle samples, 3 spleens (#25, #28, and #41) were suspect, and liver sample #30 was negative by FFPET RT-qPCR (Table 2.3). All were infected with SA01. Sample #30, not detected by FFPET RT-qPCR was also negative for RVFV antigen by IHC but positive by FFT RT-qPCR. Consequently, a plausible explanation for this negative FFPET RT-qPCR result lies in the sampling process. The FFT RT-qPCR was run on a separate frozen sample whereas IHC was run on same FFPET tissue block as FFPET RT-qPCR. RVFV causes multifocal lesions in tissues. This leads to a heterogeneous distribution of the virus in tissue samples. Therefore, considering that both the FFPET RT-qPCR and IHC results on the same sample were negative, the formalin-fixed liver sample may conceivably be negative, while the frozen sample tested by FFT RT-qPCR is positive for a low level of viral RNA (Ct 28.8-33.1). Cattle spleen sample #38 infected with Ken06, which was negative by FFT RT-qPCR but positive by IHC and FFPET RT-qPCR when 200 µl of lysate was run, could be an inverse example of this sampling disparity.

The three spleen samples (#25, #28, and #41) had FFT RT-qPCR Ct values ranging from 29 to 35 for all three gene segments. In general, samples with lower levels of viral RNA are more difficult to recover from FFPET as the yield of viral RNA from FFPET is lower than that for fresh tissues[171]. Additionally, the RT-qPCR method has a limit of detection between 10 and 100 genome copies[105] for RVFV RNA. Thus, low viral copy number could have affected the sensitivity of detection of RVFV RNA from FFPET. However, since this was not the case for corresponding SA01 infected liver (Table 2.3), the tissue type must be considered further. The spleen is a morphologically complex organ with extensions of the collagenous fibers and lymphoid follicles, which could have affected the recovery of RNA during the process. When these 3 suspect samples were re-run with increased lysate volume, one of them was detected as positive. In general, when further optimization of a protocol is conducted, the species, virus strain, and tissue type all need to be considered as their differences can affect the assay's detection sensitivity.

A combination of IHC and histopathology is the accepted tissue-based confirmatory test of choice for RVFV[77]. While the semi-quantitative liver IHC scores were consistent with FFPET RT-qPCR results (Table 2.5), in that the average Ct value for each gene segment decreased with an increase in IHC score, the histopathology results were not as well correlated. Semi-quantitative liver histopathology to FFPET RT-qPCR detection of RVFV RNA showed consistent results with the exception of 3 samples: #30, #39, #40 (Table 2.5). However, all these samples were viral antigen negative on IHC and the mild histopathology was attributed to non-RVFV related causes for #39 and #40[161]. Background inflammation due to other etiologies has been observed in the cattle and sheep used on research studies because these animals are sourced on the open market, not raised for research purposes[161].

Interestingly, #31, is another piece of liver tissue from the same SA01, 4 days post-infection calf as #30, which was positive by all assays including FFPET RT-qPCR. Whereas #31scored a 3 on histopathology, #30 only scored a 2 and although it had necrosis lesions typical of RVFV infection, it had far fewer with less than 5% of the hepatic parenchyma involved. Therefore, although this animal was definitely infected with RVFV as validated by serum titers at earlier time-points[161] and had RVF related lesions in this tissue and others, there was no viral antigen or viral RNA detected in this FFPET sample. This does not exclude the possibility of low levels of viral antigen or RNA being present. They were simply consistently outside the detection range of IHC and FFPET RT-qPCR. This highlights the need to collect multiple samples from individual animals as well as the importance of the limits of detection of our assays, something that might be overcome with a more sensitive assay technology or further assay optimization.

When FFPET RT-qPCR and IHC was compared, the sensitivity of detection of RVFV RNA by FFPET RT-qPCR was 80.9% for all non-liver samples. As per Table 2.6, FFPET IHC and PCR also correlated well for all but 6 of the 23 non-liver samples (#4, #25, #28, #29, #38, and #41). Samples #4 and #29 were kidney and the rest of the samples were spleen. IHC positive samples #25 and #38, as discussed prior, became FFPET RT-qPCR positive with use of increased volumes of lysate (Table 2.4). Interestingly, kidney sample #4, which was suspect on FFPET RT-qPCR and negative on IHC, actually had been run twice by IHC because on one run there was a rare signal in scattered glomeruli. This signal disappeared on the next block sectioning for IHC. This animal was also at 10 days post-infection when euthanized, which is a late time-point in the disease course, a time when animals that have survived the acute disease are typically no longer positive for viral antigen by IHC. Therefore, this remains a suspect

sample, a category not used for IHC results, for which these borderline samples were conservatively called negative. If it is instead a limitation of IHC's sensitivity, FFPET *in situ* hybridization for the detection of RVFV RNA, a potentially more sensitive assay, might be used to resolve the discrepancy. However, it could also be that there is residual viral RNA in the tissues, but the proteins are no longer present[172]. It is beyond the scope of the immediate study to further examine this phenomenon. Spleen sample #41 as discussed earlier has particularly low viral RNA, so a negative IHC result was unsurprising. The remaining discrepant samples (#28 and #29), spleen and kidney respectively from the SA01 infected calf with one liver sample negative for both viral antigen and RNA (#30) and a second viral antigen and RNA positive (#31), as discussed earlier, provides a good example of a lightly infected animal yielding a diversity of reads due to absence of virus in some tissue samples.

Several studies have been conducted to compare commercially available automated and manual NA extraction methods from FFPET[173–175]. Our study had similar results for both the bead and column extraction methods using 100 µl lysate for the positive/negative determination of RVFV RNA in the sample. Out of 43 sheep and cattle samples, only 3 samples (#4, #25, and #40) had inconsistent results. (Table 2.7 and 2.8). Sample #4, negative by column extraction but suspect by bead extraction, as already discussed, was a late time-point (10 days post-infection) sample with no viral antigen detected by IHC. Sample #25 was positive by column extraction but suspect by bead extraction initially. Increasing the lysate resolved this discrepancy (Table 2.4), indicating that low viral RNA was the likely cause. Sample #40, which was suspect by column extraction but negative by bead extraction was also negative by IHC and FFT RT-qPCR. Interestingly, sample #39, which is a second liver section from this KEN06 5 days post-infection calf, was negative for RVFV by all detection assays. However, these were the two tissues (#39

and #40) that scored a 1 on histopathology. Thus, while this sample is likely negative, it is not outside the realm of possibility that its viral antigen and RNA levels are lower than the current limits of detection of our assays.

Although studies have shown that using high throughput methods such as magnetic bead-based extraction methods results in high yield, reproducibility, and quantity of RNA from FFPET[173,175], we observed higher copy numbers of viral RNA using column extraction in comparison to bead extraction. Consequently, the lowest-copy number nucleic acid targets may be lost during automated nucleic acid extraction, resulting in lesser sensitivity[176]. Ali, *et al*., 2017 also reported that use of bead extraction methods can cause interference during PCR[176]. Despite this concern, overall the positive/negative determination was similar for both methods. Therefore, an automated NA extraction process, which is easy, rapid, and economic[160], could be a method of choice for detection of RVFV RNA from FFPET in comparison to the laborious manual spin column extraction process, particularly when low viral copy number is of lesser concern.

A consistent result from the same sample that is tested repeatedly is an important feature of a diagnostic test. The FFPET RT-qPCR test has an adequate repeatability (CV < 10%) for the Ct of each gene of RVFV RNA, which means the degree of variability of the data is less that 10%. We observed substantial agreement (k=0.6-0.67) between FFPET RT-qPCR for both bead and column extraction method when each of them were compared with FFT RT-qPCR using bead extraction method[167]. The substantial agreement between FFPET RT-qPCR and FFT RT-qPCR demonstrates that FFPET RT-qPCR can be used as an alternative technique for the detection of RVFV RNA. Moreover, it could be a valuable technique for retrospective studies using archival FFPET.

There were multiple limitations to our study. First, limited cross-reactivity tests were performed due to lack of availability of samples from animals exposed to a virus similar to RVFV. Only a small number of Schmallenberg virus positive samples were used to test the cross-reactivity and although Schmallenberg is a member of order *Bunyavirales* it is not an RVFV near neighbor. Second, no field samples from an RVFV endemic region were available to include in the study. Therefore, along with further cross-reactivity testing, validation testing with field samples from a RVFV endemic region should be conducted. Field samples are crucial for validation of a diagnostic test because the diagnostic sensitivity, specificity and kappa are affected by prevalence. Third, RVFV is a Select Agent in the US and research with virulent strains must be conducted in biosafety level 3 agriculture (BSL-3Ag) biocontainment facilities. This restricted the number of experimental samples that were available for this study.

Recovery of NA and determination of the presence of infectious agent from FFPET is a multi-step process. Successful extraction and detection require a standardized protocol for all steps: (1) deparaffinization, (2) NA extraction, and (3) RT-qPCR. No protocol for successful recovery of RVFV RNA from FFPET has been developed. Here, we developed a method for the detection of RVFV RNA from FFPET using a commercially available deparaffinization solution, rapid and easy bead extraction and an RVFV multiplex PCR validated on fresh and frozen tissues[152]. Amplifiable RVFV can be recovered from FFPET. This method could be used for confirmatory testing of suspected RVFV-infected formalin-fixed (paraffin-embedded) tissues and can be conducted outside of BSL-3. Furthermore, with additional analysis of quality and consequent method optimization, this technique might also be useful for retrospective research using next generation sequencing.

Acknowledgements

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Species	RVFV strain	Number of samples	Reference
Sheep	SA01	1	Faburay et al., 2016a, Faburay et al., 2016b
	KEN06	22	
Cattle	SA01	8	Wilson and Davis et al., 2016
	KEN06	12	

Table 2.1 Tissue sample sets from RVFV exposed animals

SHEEP			FORMALIN-FIXED PARAFFIN-EMBEDDED RT-qPCR				FRESH FROZEN RT-qPCR			
SAMPLE	**VIRUS**	**TISSUE**	**L (Ct)**	**M(Ct)**	**S (Ct)**	**RESULT**	**L (Ct)**	**M(Ct)**	**S (Ct)**	**RESULT**
3		liver	18.5	18.5	21.1	POS	16.0	21.6	17.1	POS
6		liver	19.3	19.5	22.1	POS	16.0	21.6	17.1	POS
11		liver	23.8	23.8	25.9	POS	19.8	24.9	20.5	POS
12		liver	23.5	23.7	26.2	POS	19.8	24.9	20.5	POS
42		liver	25.2	25.1	27.4	POS	12.2	17.0	13.2	POS
43		liver	22.1	22.7	24.2	POS	26.2	27.4	23.9	POS
5a	KEN06	liver	21.0	21.0	24.0	POS	22.0	21.0	19.0	POS
5		liver	21.7	21.5	23.6	POS	21.2	22.4	19.6	POS
21		liver	17.7	18.1	19.1	POS	17.5	18.4	15.9	POS
7		spleen	23.0	22.4	24.3	POS	17.5	22.0	17.2	POS
13		spleen	26.4	25.3	26.9	POS	21.4	26.0	21.8	POS
17		spleen	21.5	21.0	22.0	POS	18.1	23.0	18.0	POS
19		spleen	20.0	19.8	19.7	POS	20.0	20.0	16.3	POS
1	SA01	liver	30.4	30.9	33.7	POS	24.2	28.9	25.8	POS
1a		liver	29.0	30.0	30.0	POS	24.0	28.0	25.0	POS

Table 2.2 Evaluation of FFPET RT-qPCR using known positive sheep samples by FFT RT-qPCR

FFT RT-qPCR are archival data (Table 2.1); 5a and 1a are replicate samples of sample 5 and 1 respectively; RVFV gene segments: L: large, M: medium, S: small. Key: POS: positive, at least 2/3 genes Ct < 38

CATTLE			FORMALIN-FIXED PARAFFIN-EMBEDDED RT-qPCR				FRESH FROZEN RT-qPCR			
SAMPLE	VIRUS	TISSUE	L (Ct)	M (Ct)	S (Ct)	RESULT	L (Ct)	M (Ct)	S (Ct)	RESULT
23	KEN06	liver	21.4	22.0	22.5	POS	17.9	23.9	19.5	POS
24		liver	21.3	22.2	22.7	POS	17.9	23.9	19.5	POS
35		liver	21.5	21.8	23.3	POS	16.6	21.3	16.7	POS
36		liver	20.4	20.7	21.6	POS	16.6	21.3	16.7	POS
37		liver	20.5	20.8	22.6	POS	16.6	21.3	16.7	POS
37a		liver	22.0	24.0	24.0	POS	16.0	21.0	16.0	POS
22		spleen	28.0	28.1	28.6	POS	23.1	27.9	22.0	POS
32		spleen	20.8	20.5	21.8	POS	20.4	24.8	19.3	POS
33		kidney	23.8	24.1	23.9	POS	21.1	26.1	20.7	POS
34		kidney	22.9	22.9	23.1	POS	21.1	26.1	20.7	POS
26	SA01	liver	27.9	28.5	30.4	POS	27.4	32.7	28.4	POS
27		liver	28.2	28.8	30.7	POS	27.4	32.7	28.4	POS
27a		liver	29.0	29.0	30.0	POS	27.0	32.0	28.0	POS
31		liver	32.6	31.7	34.4	POS	28.8	33.1	28.8	POS
31a		liver	30.0	29.0	30.0	POS	28.0	33.0	28.0	POS
30		liver	ND	ND	ND	NEG	28.8	33.1	28.8	POS
25		spleen	ND	36.7	ND	SUS	29.0	34.2	29.0	POS
28		spleen	ND	36.2	ND	SUS	30.8	38.5	29.8	POS
41		spleen	ND	SUS	ND	SUS	31.6	33.8	30.3	POS

Table 2.3 Evaluation of FFPET RT-qPCR using known positive cattle samples by FFT RT-qPCR

FFT RT-qPCR are archival data (Table 2.1); Samples 37a, 31a, 27a are replicate samples; RVFV gene segments: L: large, M: medium, S: small. Key: ND= not detected Ct >38; POS: positive, at least 2/3 genes < Ct 38; SUS: suspect for sample, 1/3 genes < Ct 38 or at least 1/3 genes reported as suspect; NEG: negative, 3/3 genes > Ct 38

SAMPLE	HOST	VIRUS	TISSUE	FORMALIN-FIXED-PARAFFIN-EMBEDDED RT-qPCR							
				BEAD EXTRACTION (1x volume of lysate)				BEAD EXTRACTION (2x volume of lysate)			
				L (CN)	M (CN)	S (CN)	RESULT	L (CN)	M (CN)	S (CN)	RESULT
30	cattle	SA01	liver	ND	ND	ND	NEG	ND	18	ND	SUS
31		SA01	liver	85	179	1	POS	47	1,061	12	POS
39		KEN06	liver	ND	ND	ND	NEG	ND	ND	SUS	SUS
25		SA01	spleen	ND	16	ND	SUS	ND	121	2	POS
28		SA01	spleen	ND	11	ND	SUS	ND	44	ND	SUS
38		KEN06	spleen	ND	SUS	ND	SUS	8	64	SUS	POS
41		SA01	spleen	ND	SUS	ND	SUS	ND	SUS	SUS	SUS
29		SA01	kidney	ND	298	ND	SUS	ND	ND	SUS	SUS
1	sheep	SA01	liver	392	307	2	POS	297	365	16	POS
10		KEN06	lung	1,022	2657	5	POS	586	1,272	37	POS
14		KEN06	lymph node	12	1686	ND	POS	415	964	25	POS
				%Positive: 36.4%				%Positive: 54.5%			

Table 2.4 Evaluation of sensitivity of FFPET RT-qPCR in samples with low copy number

RVFV gene segments: L: large, M: medium, S: small; Key: CN: copy number; NT= not tested; ND= not detected, POS: positive; SUS: suspect; NEG: negative

SAMPLE	HOST	VIRUS	FORMALIN-FIXED PARAFFIN-EMBEDDED RT-qPCR				H Score	IHC score
			L (Ct)	M (Ct)	S (Ct)	RESULT		
23	cattle	KEN06	21.4	22.0	22.5	POS	3	2
24		KEN06	21.3	22.2	22.7	POS	3	2
35		KEN06	21.5	21.8	23.3	POS	4	3
36		KEN06	20.4	20.7	21.6	POS	4	3
37		KEN06	20.5	20.8	22.6	POS	4	3
37a		KEN06	22.0	24.0	24.0	POS	4	3
39		KEN06	ND	ND	ND	NEG	1	0
40		KEN06	ND	ND	ND	NEG	1	0
26		SA01	27.9	28.5	30.4	POS	3	2
27		SA01	28.2	28.8	30.7	POS	3	2
27a		SA01	29.0	29.0	30.0	POS	3	2
31		SA01	32.6	31.7	34.4	POS	3	1
31a		SA01	30.0	29.0	30.0	POS	3	1
30		SA01	ND	ND	ND	NEG	2	0
3	sheep	KEN06	18.5	18.5	21.1	POS	4	3
5		KEN06	21.7	21.5	23.6	POS	3	3
6		KEN06	19.3	19.5	22.1	POS	4	3
11		KEN06	23.8	23.8	25.9	POS	4	3
12		KEN06	23.5	23.7	26.2	POS	4	3
21		KEN06	17.7	18.1	19.1	POS	4	3
42		KEN06	25.2	25.1	27.4	POS	4	3
43		KEN06	22.1	22.7	24.2	POS	3	3
5a		KEN06	21.0	21.0	24.0	POS	3	3
1a		SA01	29.0	30.0	30.0	POS	3	2
1		SA01	30.4	30.9	33.7	POS	3	2

Table 2.5 Comparison of FFPET RT-qPCR detection of RVFV RNA in liver samples with histopathology and immunohistochemistry

H score is the histopathology score on a scale of 0 to 4. Similarly, IHC score is the anti-RVFV immunohistochemitry (IHC) result on a scale of 0, no detection of viral antigen, to 3, most extensive presence viral antigen. The H score and IHC score is explained in materials and method section. Samples 1a, 5a, 31a, 37a, 27a are replicate samples, H score for cattle and sheep along with IHC score for sheep are archival data (Table 2.1); RVFV gene segments: L: large, M: medium, S: small. Key: ND: not detected; POS: positive, at least 2/3 genes < Ct 38; NEG: negative, 3/3 genes > Ct 38

SAMPLE	HOST	VIRUS	TISSUE	FORMALIN-FIXED PARAFFIN-EMBEDDED RT-qPCR				IHC
				L (Ct)	M (Ct)	S (Ct)	RESULT	
33	cattle	KEN06	kidney	23.8	24.1	23.9	POS	POS
34		KEN06	kidney	22.9	22.9	23.1	POS	POS
29		SA01	kidney	ND	30.9	ND	SUS	POS
22		KEN06	spleen	28.0	28.1	28.6	POS	POS
32		KEN06	spleen	20.8	20.5	21.8	POS	POS
38		KEN06	spleen	ND	SUS	ND	SUS	POS
25		SA01	spleen	ND	36.7	ND	SUS	POS
28		SA01	spleen	ND	36.2	ND	SUS	POS
41		SA01	spleen	ND	SUS	ND	SUS	NEG
4	sheep	KEN06	kidney	ND	33.9	ND	SUS	NEG
9		KEN06	kidney	27.3	27.0	29.0	POS	POS
15		KEN06	kidney	27.8	26.5	26.9	POS	POS
18		KEN06	kidney	22.3	23.1	23.4	POS	POS
20		KEN06	kidney	24.1	24.6	24.7	POS	POS
8		KEN06	lymph node	24.4	24	25.8	POS	POS
14		KEN06	lymph node	35.5	28.4	ND	POS	POS
2		KEN06	lung	23.3	23.1	24.2	POS	POS
10		KEN06	lung	29.0	27.7	32.6	POS	POS
16		KEN06	lung	22.6	22.9	21.7	POS	POS
7		KEN06	spleen	23.0	22.4	24.3	POS	POS
13		KEN06	spleen	26.4	25.3	26.9	POS	POS
17		KEN06	spleen	21.5	21.0	22.0	POS	POS
19		KEN06	spleen	20.0	19.8	19.7	POS	POS

Table 2.6 Comparison of FFPET RT-qPCR detection of RVFV RNA with immunohistochemistry

IHC is the anti-RVFV immunohistochemistry; POS: positive, detection of viral antigen; NEG: negative, no detection of viral antigen. IHC score for sheep and cattle are archival data (Table 2.1); RVFV gene segments: L: large, M: medium, S: small. Key: NT=

not tested; ND= not detected Ct >38; POS: positive, at least 2/3 genes < Ct 38; SUS: suspect for sample, 1/3 genes < Ct 38 or at least 1/3 genes reported as suspect; NEG: negative, 3/3 genes > Ct 38

SAMPLE	VIRUS STRAIN	TISSUE	FORMALIN-FIXED-PARAFFIN-EMBEDDED RT-qPCR					
			BEAD EXTRACTION			COLUMN EXTRACTION		
			L (CN)	M (CN)	S (CN)	L (CN)	M (CN)	S (CN)
5	KEN06	liver	5.2	5.2	4.7	5.8	5.8	6.0
21	KEN06	liver	6.4	6.2	6.7	7.0	6.8	7.9
43	KEN06	liver	5.1	4.9	4.4	5.7	5.6	5.6
3	KEN06	liver	6.2	6.1	5.8	5.3	5.1	4.6
6	KEN06	liver	5.9	5.8	5.4	6.7	6.5	7.1
11	KEN06	liver	4.6	4.6	3.7	4.9	4.7	4.1
12	KEN06	liver	4.7	4.6	3.6	5.0	4.9	4.4
42	KEN06	liver	4.2	4.2	3.0	4.8	4.7	3.9
19	KEN06	spleen	5.7	5.7	6.4	6.5	6.5	7.9
7	KEN06	spleen	4.8	5.0	4.4	4.9	4.9	4.7
13	KEN06	spleen	3.8	4.1	3.2	4.0	4.1	3.5
17	KEN06	spleen	5.3	5.4	5.4	6.0	6.1	6.6
8	KEN06	lymph node	4.4	4.5	3.7	4.5	4.5	4.2
14	KEN06	lymph node	1.1	3.2	ND	3.8	3.9	3.6
20	KEN06	kidney	4.5	4.3	4.2	5.2	5.1	5.8
4	KEN06	kidney	ND	1.6	ND	ND	ND	ND
9	KEN06	kidney	3.5	3.6	2.3	3.6	3.8	3.1
15	KEN06	kidney	3.4	3.8	3.2	4.2	4.2	4.3
18	KEN06	kidney	5.1	4.8	4.8	5.8	5.6	6.2
2	KEN06	lung	4.7	4.8	4.4	5.4	5.3	5.8
10	KEN06	lung	3.0	3.4	0.7	3.9	3.9	2.7
16	KEN06	lung	4.9	4.8	5.5	5.4	5.4	6.4
1	SA01	liver	2.6	2.5	0.2	3.6	3.5	2.2

Table 2.7 Quantitative comparison of the automated magnetic bead and spin column extractions for the detection of RNA from infected sheep samples

RVFV gene segments: L: large, M: medium, S: small; Key: CN: copy number expressed as log base 10**;** ND: not detected

SAMPLE	VIRUS STRAIN	TISSUE	FORMALIN-FIXED-PARAFFIN-EMBEDDED RT-qPCR					
			BEAD EXTRACTION			COLUMN EXTRACTION		
			L (CN)	M (CN)	S (CN)	L (CN)	M (CN)	S (CN)
23	KEN06	liver	5.3	5.1	5.2	6.0	5.7	6.2
24	KEN06	liver	5.3	5.0	5.1	6.1	5.9	6.5
35	KEN06	liver	5.3	5.1	4.9	5.6	5.6	5.4
36	KEN06	liver	5.6	5.5	5.6	6.3	6.2	6.5
37	KEN06	liver	5.6	5.4	5.1	6.1	5.9	6.0
39	KEN06	liver	ND	ND	ND	ND	ND	ND
40	KEN06	liver	ND	ND	ND	ND	1.4	ND
22	KEN06	spleen	3.3	3.3	2.5	3.8	3.7	3.8
32	KEN06	spleen	5.5	5.5	5.5	6.0	5.9	6.4
38	KEN06	spleen	ND	SUS	ND	ND	1.6	ND
33	KEN06	kidney	4.6	4.5	4.6	4.9	4.9	5.0
34	KEN06	kidney	4.9	4.8	4.9	5.4	5.3	5.6
26	SA01	liver	3.4	3.2	1.7	4.1	4.0	3.7
27	SA01	liver	3.3	3.1	1.6	3.7	3.5	3.0
30	SA01	liver	ND	ND	ND	ND	ND	ND
31	SA01	liver	1.9	2.3	0.1	2.8	3.0	1.4
25	SA01	spleen	ND	1.2	ND	ND	2.2	1.0
28	SA01	spleen	ND	1.1	ND	ND	1.5	ND
41	SA01	spleen	ND	SUS	ND	ND	SUS	ND
29	SA01	kidney	ND	2.5	ND	ND	1.5	ND

Table 2.8 Quantitative comparison of the automated magnetic bead and spin column extractions for the detection of RNA from infected cattle samples

RVFV gene segments: L: large, M: medium, S: small; Key: CN: copy number expressed as log base 10**;** ND: not detected, SUS: suspect

SAMPLE	TOTAL RUNS	RVFV RNA GENE SEGMENTS	MEAN (Ct)	SD	CV (%)
17	4	L	24.76	1.49	6
		M	23.86	1.32	6
		S	26.30	2.18	8
19	9	L	22.14	1.55	7
		M	21.59	1.32	6
		S	22.57	1.27	6

Table 2.9 Inter-assay variability of threshold cycle of RVFV RNA during extraction

RVFV gene segments L: large, M: medium, S: small; SD: standard deviation, CV (%): percentage of co-efficient of variation

Diagnostic Tests	Agreement (%)	McNemar's test (p-value)	Kappa (95%CI)
IHC and FFPET RT-qPCR (B)	90.24	0.13	0.54 (0.18-0.93)
IHC and FFPET RT-qPCR (C)	92.68	0.25	0.63 (0.26-1.00)
IHC and FFT RT-qPCR (B)	90.62	1.00	0.52 (0.05-0.99)
FFPET RT-qPCR (B) and (C)	97.67	1.00	0.93 (0.79-1.00)
FFPET RT-qPCR(B) and FFT RT-qPCR (B)	87.88	0.13	0.60 (0.27-0.94)
FFPET RT-qPCR (C) and FFT RT-qPCR (B)	90.91	0.25	0.68 (0.35-1.00)

Table 2.10 Agreement between tests

Cohen's Kappa <0.2 slight agreement; 0.2–0.4 fair agreement; 0.4–0.6 moderate agreement; 0.6–0.8; substantial agreement, and >0.8 almost perfect agreement. Key: B: bead extraction; C: column extraction, FFPET: Formalin-Fixed Paraffin-Embedded Tissue; FFT: Fresh Frozen Tissue, IHC: Immunohistochemistry

Chapter 3 - Evaluation of Diagnostic Accuracy and Precision of a Competitive ELISA for Detection of Antibodies to Rift Valley Fever Virus in Cattle and Sheep Sera

Preface

This chapter is a full manuscript under review at the Journal of Virological Methods under the title:

Evaluation of Diagnostic Accuracy and Precision of a Competitive ELISA for Detection of Antibodies to Rift Valley Fever Virus in Cattle and Sheep Sera

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Introduction

Rift Valley fever virus (RVFV), a mosquito-borne, zoonotic pathogen, was first discovered in Kenya in 1930[82] and has subsequently been found in several sub-Saharan African countries[59]. The RVFV (order: *Bunyavirales*; family *Phenuiviridae;* genus *Phlebovirus*)[153] primarily affects ruminants, causing high abortion and neonatal mortality rates. Human cases have been reported with clinical signs and symptoms ranging from mild febrile illness to encephalitis and hemorrhagic fever that can lead to death[70].

Outbreaks have been reported beyond the African continent in the Arabian Peninsula[177] and the wide range of competent vectors[3] implies a risk for RVFV spread to other non-endemic countries. Moreover, the introduction of West Nile virus, another arbovirus, into North America and its ability to endure and survive in new environments[178] has raised concerns about the potential for the spread of RVFV and other arboviruses. Thus, research on rapid diagnostic techniques and implementation of surveillance programs for arboviral pathogens is crucial[4,179].

Agar gel immunodiffusion, radioimmunoassay, hemagglutinin inhibition and complement fixation are traditional serological diagnostic techniques for RVFV that are no longer used[72]. Currently, the virus neutralization test (VNT) and enzyme-linked immunosorbent assay (ELISA) are commonly used for surveillance and outbreak management[74]. Although VNT, specifically the plaque reduction neutralization test ($PRNT_{80}$), is considered the reference test, it is labor-intensive, time consuming, expensive, and requires virus appropriate biocontainment. As an alternative to VNT, ELISA offers a safe, reliable, and efficient technique for the detection of antibodies against RVFV. Several ELISA formats have been developed and validated using cell lysate and purified viral antigens[87,91,94,180,181]. However, production and purification of viral antigens require a biocontainment facility with high production costs and risk of incomplete

inactivation of the virus[182]. In contrast, recombinant nucleoprotein (recNp) based ELISA does not require a biocontainment facility for its production or use, thus eliminates the human risk associated with diagnostics[88]. Additionally, nucleocapsid protein is a highly immunogenic and abundant viral component[183,184]. Several ELISAs based on recNP have been developed and used[88,185–188]. Thus, recNp is a suitable diagnostic antigen for ELISA. Along with being non-infectious and stable, recNp is easier, cheaper, and safer to produce and purify[187,188]. Here we examine the suitability of a recombinant nucleoprotein based cELISA that was developed for potential inclusion in the USDA Animal and Plant Health Inspection Service (APHIS) National Veterinary Stockpile.

The objective of the study was to determine the diagnostic sensitivity and specificity of a recNp based competitive ELISA (cELISA) assay to detect RVFV antibodies, using sera samples from cattle and sheep that were experimentally infected with a candidate RVFV vaccine or virulent RVFV strain, as well as using known RVFV negative sera, by comparing them with paired $PRNT_{80}$ results.

Materials and Methods

Samples

All available samples from the archived study sample sets detailed in Table 3.1 were included in this study. A total of 165 sera that includes (cattle = 53, sheep = 96) from ruminants experimentally inoculated with the MP-12 RVFV vaccine and/or a wild-type RVFV strain (Saudi Arabia 2001 (SA01), Kenya 2006 (Ken06) or ZH501) as well as in room mock-inoculated animals' sera (cattle = 11, sheep = 5), previously demonstrated to be negative for RVFV antibodies, were used[126,155,161,189]. All these prior animal studies were conducted in biosafety

level 3 agriculture (BSL-3Ag) biocontainment facilities in Kansas except the study by (Weingartl et al., 2014), which was conducted in a zoonotic BSL-3Ag biocontainment in Manitoba, Canada.

Additionally, a set of known RVFV negative sera samples (cattle = 330 and sheep = 179) were collected from ruminants unexposed to RVFV. All samples were obtained from animals born and raised in the United States. All the sera were heat inactivated by adding 2.5% Tween-20 at a dilution of 1:10 to the serum and heating samples at 60°C in a water bath for 2 h[161]. Serum from BSL-3Ag were safety tested by demonstrating no cytopathic effects after three blind passages in susceptible cell cultures.

cELISA

The cELISA was performed according to the manufacturer's instructions (Veterinary Medical Research & Development (VMRD), Washington, USA) for the detection of antibodies against RVFV in sera. Briefly, 50 µL of serum per sample were loaded into the recombinant RVFV nucleoprotein antigen coated 96-well plate and incubated for 2 h at room temperature (RT) (23 ± 2°C). After 5 washes, 50 µL of the primary antibody, monoclonal anti-RVFV nucleoprotein antibody, were added to the plate and incubated for 30 min at RT (23 ± 2°C). After 5 washes, 50 µL of polyclonal horseradish peroxidase conjugated anti-mouse serum were added and incubated for 30 min at RT. The plate was washed 5 times, 50 µL of tetramethylbenzidine (TMB) substrate solution added, plates were incubated away from direct light for 15 min and 50 µL of ready-to-use TMB Stop solution was added. All washes consisted of 1x RVFV solution prepared according to the manufacturer's instructions. Readings were recorded immediately after the addition of 50 µL of stop solution per well on an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., VT, USA) at 450nm. The sample mean optical densities (OD) were converted into a percentage inhibition (% I) using the equation: 100 x [1 - (sample OD ÷

negative control OD)]. According to the manufacturer, the test is valid if the mean of the negative control OD is > 0.4 and < 1.5. Samples were run in duplicate.

$PRNT_{80}$

The $PRNT_{80}$ methods and RVFV serum sample dataset used in our analysis are already published[85]. Briefly, two-fold serial dilutions of sera samples, from 1:10 to 1:1280, were carried out using a Minimum Essential Medium (MEM) (Thermo Fisher Scientific, Grand Island, NY) mixed with an equal volume of diluted RVFV MP-12 virus forming 50 PFU/250µL per well of a 96-well plate, incubated at 37°C for 1 h and inoculated onto a confluent monolayer of Vero cells plated on 12-well plates. After 1 h of incubation at 37°C with periodic plate rocking, MEM containing 1% methylcellulose overlay was added. After 5 days of incubation at 37°C, plaque formation was quantified after incubation with 0.5% crystal violet fixative stain for 1 h at RT. Neutralizing antibody titers were calculated as the reciprocal titer of the highest serum dilution at which the number of plaques is reduced by 80% or more compared to the MP-12 strain virus control.

Statistical Analyses

Linearity and analytical specificity

Linearity of the cELISA assay was determined using 10-fold serial dilutions of RVFV known antibody-positive sheep sera run in duplicate. The correlation between each dilution and the % inhibition for the cELISA assay was established. Analytical specificity was determined using a total of 509 known RVFV negative sera run in duplicate.

Diagnostic Accuracy

We determined the optimal cut-off value that would optimize the sensitivity and specificity of the cELISA test in this set of samples, by comparing it to the results of the

reference test ($PRNT_{80}$) based on two cut-offs, using a receiver operating characteristic (ROC) curve. The area under the curve (AUC) for each comparison was computed. Moreover, the sensitivity and specificity of the cELISA test were computed, using both the manufacturer and the optimal cut-off values, relative to the results of the $PRNT_{80}$ test, considered the reference test.

Repeatability of the cELISA assay

Positive and negative controls from the cELISA kit were used to calculate the coefficient of variation (CV= standard deviation/mean) using the manufacturer provided cut-off values for the test. The mean ODs of the negative control that were run in triplicate and positive control that were run in duplicate from seven cELISA plates were used to assess the inter-assay repeatability of the cELISA assay. The tests were performed on different days at room temperature between 22-24°C by the same operator.

Agreement between tests

The Cohen's Kappa statistic and the McNemar's test for paired data were used to determine the agreement beyond due to chance and to test the proportion of positive results obtained between the cELISA test and the $PRNT_{80}$, respectively. The scale of Landis and Koch [167] was used to interpret the agreement between tests, as follows: <0 poor agreement, 0.01 to 0.2 slight, 0.21 to 0.40 fair, 0.41 to 0.6 moderate, 0.61 to 0.80 substantial and 0.81 to 1.0 almost perfect agreement. A non-significant McNemar's test ($P > 0.05$) indicates there is little evidence that the proportion of positives differ between tests[168]. Statistical analyses were conducted using STATA 12 software (StataCorp LP, Texas, USA).

Results

Linearity of the cELISA assay

The linear correlation analysis was established between each dilution of RVFV known antibody-positive sheep sera and the % inhibition for the cELISA assay (Fig. 1). An R^2 of 0.98 showed that this assay is precise across a large dynamic range.

Specificity test using known negative samples

The specificity of the cELISA assay was determined to be 99.2% (95% CI = 98.0%-99.8%) using known RVFV negative sera. Out of 509, 4 samples were detected as false positives. Additionally, all in room mock-inoculated animals' sera was determined to be negative by cELISA, which is in concordance with the results obtained previously[155,161].

Diagnostic sensitivity and specificity of cELISA and assessment of agreement between tests

In addition to determining the diagnostic sensitivity and specificity using the manufacturer provided cut-off of 60%, we determined optimal cut-offs that would optimize the sensitivity and specificity of the cELISA. The cELISA cut-offs were obtained using an ROC approach by comparing it to the results of the $PRNT_{80}$ test at two different cut-offs: <1:10 and 1:40. Two $PRNT_{80}$ cut-offs were used in order to include the lowest and highest cut-off used for $PRNT_{80}$[85,155,161,178]. cELISA cut-offs expressed as a % inhibition (%I) of 46% and 68% were obtained when compared to $PRNT_{80}$ results based on cut-off values of <1:10 and 1:40, respectively. The AUC for cELISA was 0.98 when compared to $PRNT_{80}$ at cut-offs <1:10 and 1:40 (Fig 2). The sensitivity and specificity of the cELISA test, computed using different cut-off values are reported in Table 3.2.

Based on the McNemar's test, the proportion of positives obtained by cELISA at cut-offs 60%, 46% and 68% compared to $PRNT_{80}$ at cut-offs <1:10 and 1:40 did not significantly differ (P>0.05). The extent of agreement between the tests beyond chance was considered almost perfect according to the (Landis and Koch, 1977) scale (range = 0.83 to 0.89) (Table 3.2).

Repeatability of cELISA assay

The overall mean OD was 0.07 with a standard deviation of 0.01 for the positive control and 0.95 with a standard deviation of 0.16 for the negative control. Inter-assay repeatability, expressed as a percentage of the coefficient of variation (CV) between those means, were 13% and 17% for positive and negative controls, respectively.

Detection of antibodies by the cELISA and $PRNT_{80}$ test over time

Detection of antibodies from sheep and cattle challenged with different RVFV strains by the cELISA at cut-off 60% and the $PRNT_{80}$ at cut-off 1:40 was compared. RVFV antibodies were detected as early as 5 and 6 DPI in cattle and sheep samples, respectively, by both the cELISA and $PRNT_{80}$ (Table 3.3). However, variability in the detection of antibodies was observed between cELISA and $PRNT_{80}$ in both cattle and sheep. The cELISA detected antibodies in 2/4 of cattle samples in comparison to no detection by $PRNT_{80}$ by 5 DPI. Also, the cELISA detected antibodies in a larger number of samples by 5, 6, and 7 DPI in both cattle and sheep in comparison to $PRNT_{80}$ (Table 3.3, asterisked entries). In contrast, $PRNT_{80}$ showed a higher sensitivity in later days (10 DPI) compared to cELISA in cattle samples. Since the $PRNT_{80}$ was considered the reference test, samples that tested positive by cELISA but negative by $PRNT_{80}$were considered false positives for cELISA.

Discussion

Rift Valley Fever (RVF), an emerging mosquito-borne disease that causes losses of thousands of livestock and millions of dollars during outbreaks in endemic areas, spread beyond its endemic zone to the Arabian Peninsula in 2000[54,65]. Its further spread would have devastating societal effects, impacting both animal and human health and causing significant economic losses. Therefore, it is of utmost importance to develop and validate an efficient, economical, rapid and easy diagnostic test for early diagnosis and surveillance of RVF that will help diminish the disease burden. Here we highlight the advantages of a prototype cELISA developed for the USDA APHIS National Veterinary Stockpile that is simple, rapid, reliable, and cost-effective compared to the VNT method.

Selection of an optimal cut-off is crucial when performing a test in order to increase the discriminatory power between infected and non-infected samples and to avoid error. Cut-offs are selected for the intended application on the basis of disease prevalence, predictive values, and costs[190]. Different cut-offs can be used for testing in non-endemic vs. endemic countries. For instance, in order to reduce the number of false positive results, the cut-off can be increased such that there is an increase in specificity of the test. When testing uninfected animals especially in non-endemic regions during surveillance, the cut-off can be lowered so that the number of false negative results will decrease, increasing the sensitivity of the test. Similarly, when performing the confirmatory test in epidemic countries, the cut-off can be increased in order to decrease the false positive results thereby increasing the specificity. Other factors such as origin and biological differences among the animals tested should be taken into consideration when determining the optimal cut-off[181,191]. Moreover, geographic area, genetics, nutritional status, and stage of infection can also impact the diagnostic sensitivity and specificity of the test[192]. Using

the cut-off of 60% for cELISA and 1:40 for $PRNT_{80}$, there was no significant association between origin of samples (cattle vs sheep) with the sensitivity (P = 0.86) and specificity (P = 0.44) of the cELISA test (data not shown).

Diagnostic tests should produce consistent results when tested repeatedly on the same sample. The degree of variability of the data of the prototype cELISA is less than 20%. Moreover, there was almost perfect agreement beyond chance between the $PRNT_{80}$ and cELISA (k > 0.8)[167]. These results support that this prototype cELISA is comparable to $PRNT_{80}$, thus could be used as an alternative serological diagnostic test for the RVF.

Antibodies against RVFV start to develop within 4-7 days of infection[74]. One prior study, however, showed the detection of antibodies against RVFV using recNp based cELISA at 9-11 DPI[193]. The detection of antibodies as early as 5 and 6 DPI by cELISA in our study, demonstrates its applicability in early detection of antibodies against RVFV. Antibodies from 6 samples from experimentally challenged sheep and cattle that tested positive by the cELISA were not detected by $PRNT_{80}$. Since ELISA detects antibodies against all viral components, it could be more sensitive than $PRNT_{80}$ that is specific for detection of viral neutralizing antibodies only[89].

Our research was limited in that no cross-reactivity tests were performed due to non-availability of serum samples from animals exposed to a virus similar to RVFV. In addition to cross-reactivity testing, further validation testing with field samples from a RVFV endemic region should also be conducted. In the US, RVFV is a Select Agent and research with virulent strains must be conducted in biosafety level 3 agriculture (BSL-3Ag) biocontainment facilities. This restricted the number of experimental samples that were available for this study.

Despite these limitations, we conclude that the prototype cELISA has the potential to be a useful assay for diagnosis and surveillance of RVFV. It can detect antibodies to RVFV in both vaccinated and experimentally challenged sheep and cattle. It offers a safe alternative technique to classical serological methods of detection of antibodies against RVFV that does not require live virus or viral inactivation, and because it is based on recombinant protein, its reagents can be produced outside high containment[151]. Although a commercial, multispecies cELISA for the detection of antibodies against RVFV already exists (IDVet Innovative Diagnostics, Grabels, France), it is not approved for use in the US. This alternative cELISA is a promising alternative.

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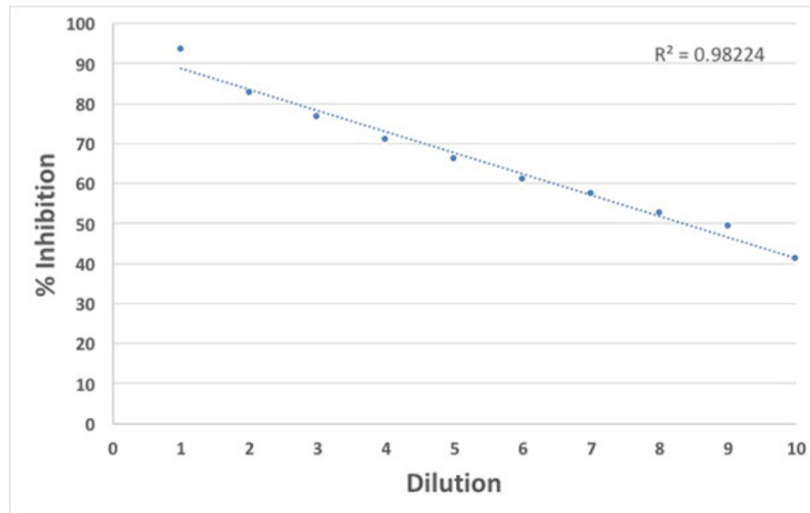

Figure 3.1 Example of the typical linearity of this cELISA assay.

10-fold serial dilutions of RVFV antibody positive sheep serum were run in duplicate. Each dot represents the mean of the OD at a given dilution. An R^2 of 0.98 shows the precision of this assay across a large dynamic range.

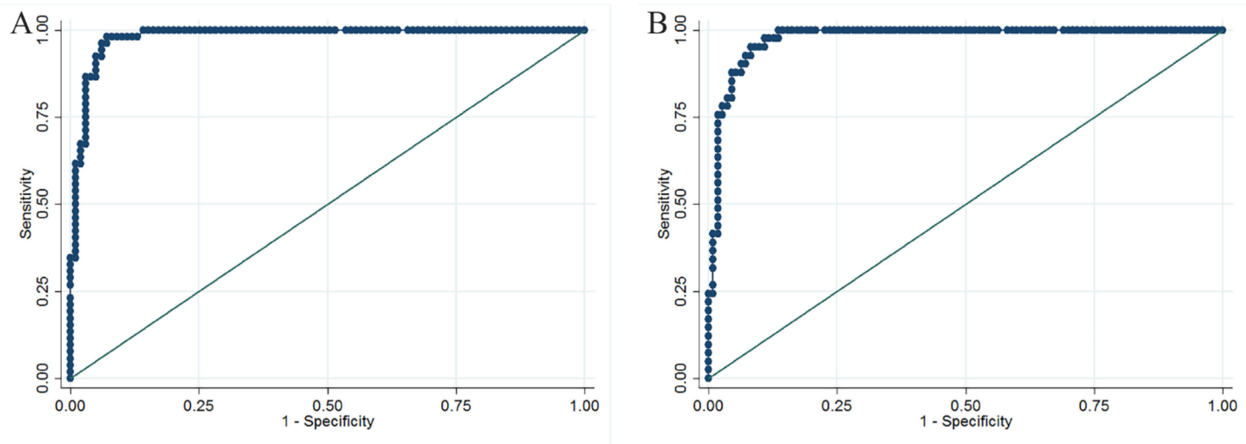

Figure 3.2 Receiver operating characteristic analyses of the cELISA for sheep and cattle serum samples.

(A) Diagnostic accuracy was calculated from experimental sera determined positive or negative by $PRNT_{80}$ at cut-off <1:10 and the Area Under Curve (AUC) was 0.98.

(B) Diagnostic accuracy was calculated from experimental sera determined positive or negative by $PRNT_{80}$ at cut-off 1:40 and AUC was 0.98.

Species	RVFV strain	Number of samples	Reference
Sheep	ZH501	4	Weingartl et al., 2014
Cattle		4	Weingartl, unpublished
Sheep	MP-12	74	Wilson et al., 2014
Cattle		14	
Sheep	SA01*	8	Faburay et al., 2016
	KEN06*	10	
	Mock-inoculated	5	
Cattle	SA01	18	Wilson et al., 2016
	KEN06	17	
	Mock-inoculated	11	

* Saudi Arabia 2001 = SA01; Kenya 2006 = KEN06

Table 3.1 Serum sample sets from RVFV exposed animals

$PRNT_{80}$ cut-off	cELISA (% inhibition)	Sensitivity[a]% (95% CI)	False Negative Rate[c] %	Specificity[b]% (95% CI)	False Positive Rate[d] %	McNemar's test (*P* value)	Kappa (95% CI)
<1:10	60% (manufacturer)	86.5 (74.2-94.4)	13.5	97.0 (91.4-99.4)	3.0	0.34	0.85 (0.76 - 0.94)
	46% (optimum)	96.2 (86.6-99.5)	3.8	93.9 (87.3-97.7)	6.1	0.29	0.89 (0.81- 0.96)
1:40	60% (manufacturer)	95.1 (83.5-99.4)	4.9	91.8 (85.0-96.2)	8.2	0.06	0.83 (0.73-0.92)
	68% (optimum)	87.8 (73.8-95.9)	12.2	95.5 (89.7-98.5)	4.5	1.00	0.83 (0.73 - 0.93)

Table 3.2 Diagnostic sensitivity and specificity of cELISA compared to $PRNT_{80}$ using cut-offs and assessment of their agreement

A total of 151 sera samples were used to determine the sensitivity and specificity of the cELISA at each % inhibition.

[a,b]Sensitivity was calculated as TP/(TP+FN), specificity was calculated as TN/(TN+FP), where TP is the number of true positives; FN is the number of false negatives, TN is the number of true negatives, and FP is the number of false positives. [c,d]False negative rate was calculated as FN/(TP+FN) and false positive rate was calculated as FP/(TN+FP).

Days post inoculation (DPI)	Sheep (#positive/total tested)		Cattle (#positive/total tested)	
	$PRNT_{80}$	cELISA	$PRNT_{80}$	cELISA
0	0/9	0/9	0/10	0/10
1	0/9	0/9	-	-
2	0/7	0/7	0/2	0/2
3	0/9	0/9	0/1	0/1
4	0/4	0/4	0/3	0/3
5	2/6	2/6	0/4*	2/4*
6	1/5*	3/5*	1/4*	3/4*
7	1/3*	3/3*	4/4	4/4
8	3/3	3/3	-	-
9	2/2	2/2	-	-
10	5/6	5/6	5/6*	4/6*
14	2/2	2/2	2/2	2/2
21	2/2	2/2	1/1	1/1
27	2/2	2/2	-	-
28	3/3	3/3	2/2	2/2
20	-	-	-	-
37	-	-	2/2	2/2
38	-	-	1/1	1/1

-: No samples were available for testing; *: Highlights differences between the cELISA and the $PRNT_{80}$ results

Table 3.3 Comparison of cELISA with $PRNT_{80}$ in detecting antibodies in sera from sheep and cattle challenged with different strains of RVFV as per Table 3.1

Chapter 4 - Conclusion

Rift Valley Fever is a transboundary disease of significant global concern[70,74,194]. The threat of its re-emergence in endemic countries and emergence in non-endemic countries increases with the globalization of animal trade and climate change. There is a defined need for the development of diagnostic tests for the detection of RVFV[70,152]. The research presented in this thesis extends the diagnostic assay options available for this important disease.

First, we developed a novel extraction method for the detection of RVFV RNA from FFPET using RT-qPCR. This method expands routine diagnostics, surveillance, and molecular epidemiological studies to the use of inactivated RVFV samples as opposed to samples containing live virus, whose handling requires significantly more biosafety precautions. Furthermore, this methodology could be expanded to include detection of additional nucleic acid targets for other high impact pathogens or used for transcriptional studies that must rely on archival FFPET.

Second, we evaluated a prototype cELISA specifically developed for the USDA APHIS National Veterinary Stockpile. Since ELISA tests for RVFV are currently not available in the US, we believe that the development and evaluation of this cELISA is of great value to the US agriculture as well as to the research community. We highlighted the advantages and limitations of this prototype cELISA. The next logical next steps are testing the cELISA with field samples from an RVFV endemic region and a specificity assessment using RVFV nearest neighbor virus infected serum samples.

Both of the assays described in this thesis are in the early stages of validation. According to the OIE guidelines for diagnostic assay development[6], these assays are at stage 2 validation except that the analytical sensitivity and specificity determination for this cELISA assay, part of

validation stage 1, which was conducted by another research team, is not yet published. Both assays need further validation in endemic countries for completion of stage 2. After completion of all four validation stages, these diagnostic tests could be used in endemic as well as non-endemic countries and continuous monitoring of their performance can be established.

Taken together, this research evolves the field of innovative, sensitive, and safe diagnostic tests for RVFV. In the longer run, the application of these diagnostic tests will help in the detection of RVF and thus, the timely application of control measures. Additionally, the FFPET RVFV RNA detection methods could be adapted for use with other RNA viruses and are useful for retrospective infectious diseases research based on archival FFPE tissues. The diagnostic assay validation approaches used for both assays are applicable to other diagnostic assay development efforts. In conclusion, this research contributes to the field of infectious diseases diagnostic assays and its methods have broader applications.

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